FANCONI ANEMIA SIGNALING: THE ROLE OF FANCD2 DURING M PHASE

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Abstract

In 1927, Guido Fanconi described a hereditary condition presenting panmyelopathy accompanied by short stature and hyperpigmentation, better known as Fanconi anemia (FA). With this discovery, the genetic and molecular basis underlying FA has emerged as a field of great interest. FA signaling is critical in the DNA damage response (DDR) to mediate the repair of damaged DNA. This has attracted a diverse range of investigators, especially those interested in aging and cancer. However, recent evidence suggests FA signaling also regulates functions outside of the DDR, with implications in many other frontiers of research. The majority of research regarding FA signaling and the cell cycle primarily investigates DNA damage repair and its role during S phase and replicative stress. Here we discuss the relevant roles of FA signaling and FANCD2 during M phase and its particular role in chromosome segregation, along with a novel FANCD2 interacting partner.

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List of Abbreviations

ΔNp63	Tumor protein p63
A	Adenine
AA	Amino acid
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and Rad3 related
BRCA	Breast cancer susceptibility gene
BLM	Bloom syndrome protein
C	Cytosine
cDNA	Complementary DNA
Cdk-1	Cyclin-dependent kinase 1
CENP-E	Centromere protein E
CIP	Calf intestinal
CON	Control samples
CRL	Colonic epithelial cells
d	Day
DEB	Diepoxybutane
DDR	DNA damage response
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol

EIF4A1	Eukaryotic translation initiation factor 4A1
EML4	Echinoderm microtubule-associated protein like 4
EV	Empty Vector
FA	Fanconi anemia
FA/BRCA	The canonical FA signaling pathway
FAAP	FA associated protein
FACS	Fluorescent activated cell sorting
FAN1	FANCD2/FANCI associated nuclease 1
FANC	Fanconi anemia complementation group
FANCD2-L	FANCD2 monoubiquitination
FANCD2-S	Non-ubiquitinated FANCD2
FBS	Fetal bovine serum
G	Guanine
G_0	Gap 0
G_1	Gap 1
G_2	Gap 2
GFP	Green fluorescent protein
GOF	Gain of Function
h	Hour
HDAC3	Histone deacetylase 3
HJ	Holiday junction
HR	Homologous recombination

HSP90	Heat shock protein 90
ICL	Interstrand crosslink
IF	Immunofluorescence
IP	Immunoprecipitation
IR	Irradiation
kDa	Kilodalton
kJ	Kilojoule
Lys	Lysine
m	Meter
M	Mitosis
min	Minute
MMC	Mitomycin C
MMS	Methyl methane sulfonate
MMR	Mismatch repair
mt	Mutant type
mRNA	Messenger RNA
MT	Microtubule
NER	Nucleotide excision repair
NHEJ	Non-homologous end joining
nud	Nuclear distribution gene
NUDC	Nuclear distribution protein C
PBS	Phosphate-buffered saline

PCR	Polymerase chain reaction
PLK1	Polo-like kinase 1
PMSF	Phenylmethylsulfonyl fluoride
PSMC2	Protease regulatory subunit 7
PVDF	Polyvinylidene fluoride
RBMX	RNA binding motif protein, X-linked
RCC1	Regulator of chromosome condensation 1
RFP	Red fluorescent protein
RNA	Ribonucleic acid
s	Second
S	Synthesis
shRNA	Short hairpin RNA
T	Thymine
Thr	Threonine
TLS	Translesion DNA synthesis
U2OS	Human bone osteosarcoma epithelial cells
Ub	Ubiquitin
UVC	Ultraviolet C
VASP	Vasodilator-simulated phosphoprotein
w	Week
WB	Western blot
wt	Wild type

Chapter One: Literature Review

1.1 Fanconi Anemia

Fanconi anemia (FA) is a rare autosomal recessive disease affecting approximately 1 of every 136,000 newborns [1]. Originally described by the Swiss pediatrician in 1927, Guido Fanconi, today FA provides insight into a number of biological mechanisms and medical conditions. Clinically, FA contributes to numerous health complications, including the early onset of aging, multi-organ congenital defects, bone marrow failure leading to pancytopenia, and a remarkably high predisposition to hematological and non-hematological malignancies [2]. Cells derived from FA patients display distinct patterns of chromosomal abnormalities, presenting *tri-* and *quadri-*radial figures in the chromosome spread. Additionally, FA cells demonstrate deficiencies in DNA damage repair and are characterized by genome instability and hypersensitivity to DNA crosslinking agents, such as mitomycin C (MMC), diepoxybutane (DEB), and cisplatin [3].

In the pursuit to understand FA and its symptoms, FA has become a unique genetic model system to study cancer etiology, especially in the field of DNA damage [4-6]. The cellular and organic changes shown in FA patients suggest that the signal transduction pathway(s) underlying FA may regulate organ development. Furthermore, FA-associated symptoms suggest that FA signaling acts as a regulatory network in governing a broad range of biological processes beginning at embryogenesis and progresses throughout the patient's lifespan.

1.2 The FA Genes

To date, twenty-two FA complementation groups (Table 1) have been described (FANCA /B /C /D1 /D2 /E /F /G /I /J /L /M /N /O /P /Q /R /S /T /U /V & /W) [1, 7-9]. All of these groups have been identified based upon biallelic germline mutations that cause the FA phenotype, with the exception of FANCB and FANCR (Rad51) [10, 11]**.** In trying to understand the nature of FA and its symptoms, many studies have shown that the FA genes and pathways are perturbed.

Subtype	Alias	FA patients (~ %)	Chr. Location	Protein Product (Kd)	Known Key Features of the Protein	Ub
A	FANCH	64	16q24.3	163	Core complex, Phosphorylated	+
B		2	Xp22.2	95	Core complex	+
C		12	9q22.3	63	Core complex	+
D1	BRCA2	2	13q12-13	380	HR	-
D2		4	3p25.3	155, 162	ID complex, monoubiquitinated, incision, TLS, HR, S phase arrest	+
E		1	6p21-22	60	Core complex	+
F		2	11p15	42	Core complex	+
G	XRCC9	8	9p13	68	Core complex	+
I		1	15q25-26	150	ID complex, phosphorylated, monoubiquitinated	+
J	BACH1, BRIP1	2	17q22-24	130	RecQ DEAH helicase family, HR, MMS, TLS, DSB repair	-
L	POG, PHF9	0.4	2p16.1	43	Core complex, the ubiquitin ligase (E3)	+
M		0.1	14q21.3	250	DNA translocase activity, lesion recognition, core complex	+
N	PALB2	0.7	16q12.1	130	HR, DSB repair	-
O	RAD51C	0.1	17q25.1	47	RAD51 paralog, HR,	-
P	SLX4, BTBD12	0.5	16p13.3	200	Scaffold protein, endonuclease, unhooking crosslink, TLS, Telomere maintenance	-
Q	ERCC4, XFP	0.1	16p13.12	101	Endonuclease, NER	-
R	RAD51	0.1	15q15.1	45	HR	-
S	BRCA1	0.1	17q21.31	220	HR	-/+
T	UBE2T	<0.1	1q32.1	22.5	Ubiquitin-conjugating enzyme (E2); NER	+
U	XRCC2	<0.1	7q36.1	34	Involved in HR, Resolving D-loop structure	-
V	REV7, MAD2L2	<0.1	1p31	24	Subunit DNA polymerase ζ involved in TLS	-

W	RFWD3	<0.1	16q23.1	~90	The ubiquitin-protein ligase (E3)	-/+

Table 1.1 Overview of the twenty-two Fanconi anemia complementation groups. Ub: Ubiquitination (required for monoubiquitination); DSB, double strand break; HR, Homologous recombination; ID complex, FANCD2-FANCI; MMR, mismatch repair; NER nucleotide excision repair; TLS, translesion DNA synthesis.

1.3 The FA/BRCA Pathway

Long standing evidence suggests that a common signaling pathway acts to prevent the manifestation of FA and FA-like phenotypes. Comprised of at least twenty-two FA gene-encoded proteins (Table 1), the aforementioned signaling pathway has been coined the FA pathway [12, 13]. Additionally, this pathway is commonly termed the FA-BRCA pathway, as several FA genes also encode breast cancer (BRCA) susceptibility gene products (Table 1).

Although the presentation of FA varies, dependent upon which FA gene(s) is mutated, the notion of a common signaling pathway involving the FA proteins is supported by the similarities in the clinical symptoms displayed throughout the FA-subtypes [1, 14]. Currently, in addition to the twenty-two FA proteins, the FA pathway consists of a number of FA associated proteins such as FAAP20/24/100, MHF1/2 (FAAP16/10) [15, 16], and numerous interacting partners including FAN1 [17-20], DNA polymerase eta [21] and REV1 [22].

The canonical FA signaling pathway is often dissected into three parts. Part I, comprises the FA core complex along with FANCT (ubiquitin conjugating enzme-E2) and upstream regulators. The core complex mainly acts as an ubiquitin ligase-E3, utilizing FANCL as the catalytic unit to monoubiquitinate FANCD2 and its paralog FANCI at Lys561 and Lys523, respectively [23]. Thus, Part I consists of FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL, FANCM, FANCT, FAAPs and others [24, 25] (Figure 1.1). Part II, the FA ID complex is comprised of FANCD2 and FANCI (Figure 1.1). Part III, the functional units downstream of Part II, contains DNA repair proteins that act in coordination following the activation/monoubiquitination of FANCD2/FANCI (Figure 1.1).

The pathway is comprised of helicases (FANCM/J), nucleases and/or their collaborators (FANCQ/ or P), other enzymatic proteins (FANCL/T/V for E3, E2 and polymerase activities respectively), complex/scaffold proteins (FANCA/B/C/D2/E/F/G/I/P), as well as proteins involved in specific DNA damage repair processes.

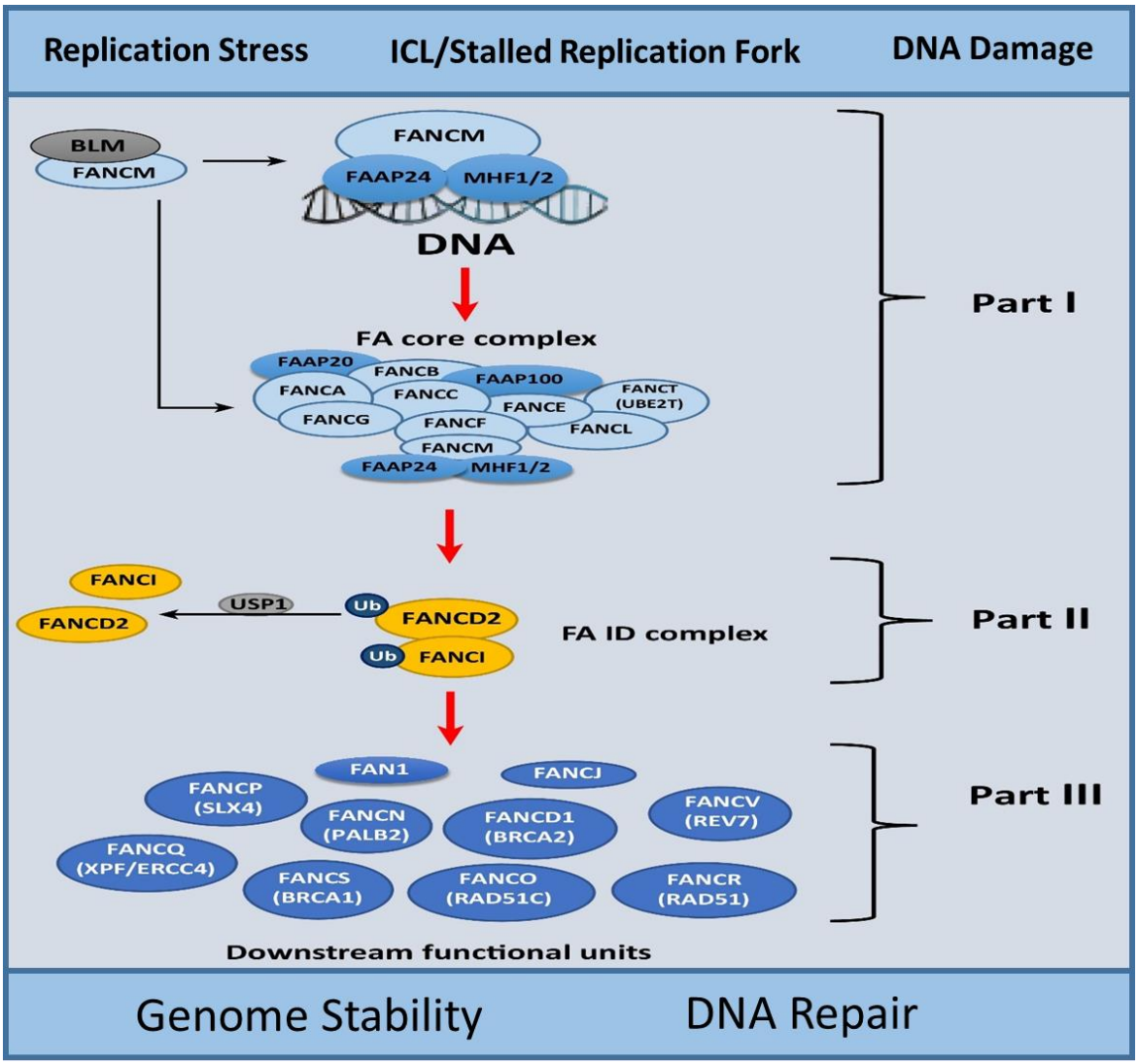


Figure 1.1. Schematical representation of the canonical FA pathway. Activation of the FA/BRCA pathway can occur subsequently after replication stress, ICL, stalled replication forks, DNA damage and more to maintain genome stability or repair DNA damage. Part I) the FA proteins (FANCA, B, C, E, F, G, L, M, T and possibly I) along with FAAPs (FAAP 20/24/100 and MHF1/2) form the FA core complex. Part II) FANCD2 and its paralog FANCI, comprise the heterodimer FA ID

complex. Part III) downstream of Part-II, the remaining FA proteins. Monoubiquitinated FANCD2 and FANCI can be deubiquitinated by USP1, thereby inactivating the pathway. Red arrows indicate the canonical FA pathway. Ub indicates monoubiquitination.

1.4 FA Signaling and DNA Damage

Constant exposure to endogenous and exogenous genotoxic agents can compromise genome stability, when the DNA damage response (DDR) is compromised [26]. Checkpoint mechanisms serve as a major regulatory function in governing the DDR and ensure the coordination of DNA repair proteins, which detect and repair DNA damage to protect cells from genome instability [26]. In these checkpoint systems, the activation of the ataxia telangiectasia-mutated (ATM) and ATM and Rad3-related (ATR) DNA repair pathways are well-recognized master responses to genotoxic stresses [27]. In the event of DNA damage, repair proteins perform various roles by sensing damaged DNA and repairing it. Alternatively, repair proteins initiate processes to eliminate damaged cells [28, 29]. The canonical FA pathway has been identified as an essential part of the DDR, and can be activated upon DNA damage, especially from DNA crosslinks or during DNA replication [30-34].

Studies have consistently conferred that when the FA pathway is impaired, cells are hypersensitive to DNA damage, and unable to successfully repair damaged DNA. Following DNA damage, the FANCM-FAAP24-MHF complex has been shown to act upstream of the DDR as key components along with ATR in the detection of DNA damage, which initiates the signaling transduction pathways to promote the monoubiquitination of FANCD2 and its paralog FANCI [35-37]. Earlier studies reporting ATM-dependent phosphorylation at S222 of FANCD2 provided further evidence for the role of FANCD2 in the DDR, especially in the S-phase checkpoint response [38, 39].

FA signaling acts to promote all known mechanisms of DNA repair, which include DNA interstrand crosslink (ICL) repair, homologous recombination (HR), nucleotide excision repair (NER),

translesion synthesis (TLS) repair, and Holiday junction (HJ) resolution [40]. FANCD2 can also regulate and cooperate the function of nucleases, enzymes responsible for unhooking the crosslinks in the early phase of DNA crosslink repair such as FANCP (SLX4) [41] and FAN1 [41, 42]. Recently, crosslink repair has also been implicated in the involvement of NER, with the emergence of FANCQ (XFP/ERCC4) and novel functions of FANCM and FANCT [7, 43-45]. In the later phases of crosslink repair, HR and/or non-homologous end joining (NHEJ) is proposed to correct errors left or generated from TLS to fully ensure genome integrity [32]. Although NHEJ is not as accurate as HR in maintaining the integrity of the genome, both repair mechanisms are essential and act in synergy, involving FANCS, FANCO, FANCD1/2 [46, 47]. Furthermore, FANCD2 appears to also provide a platform for DNA repair proteins to function, such as in the case of CtIP in ICL repair [48]. Moreover, FA signaling modulates the function of proteins involved in mismatch repair (MMR) that do not directly participate in the course of DNA crosslink repair [49].

1.5 Multifaceted Nature of FA Signaling

FA signaling has been implicated in a number of regulatory processes from embryogenesis to aging [50]. Increasingly, with the continuous identification of interacting proteins and pathways, both crosstalk and regulatory functions are highly influential to achieve multiple FA signaling functions [7, 51]. Within the activation of FA signaling, numerous players have been recently recognized for their contributions, including Rad6 [52], Rad18 [52-54], BLM [55, 56], UHRF1 [57], MITF [58], Aurora A [59] and many more. Evidenced by crosstalk, research into FA has continually demonstrated that FA signaling may act as a regulatory network, in addition to the primary FA-BRCA pathway [7].

The canonical FA pathway (FA/BRCA pathway) is most commonly recognized for its roles in DNA damage repair. In contrast, non-canonical FA signaling independent of the FA/BRCA pathway have been reported to promote both DNA damage repair as well as roles independent from repair (Table 1.2) [50]. Currently, it is widely acknowledged that the FA and FA-associated proteins, such as FANCC, FANCA [60], FANCJ [61], FAAP24 [62], FANCI [63], etc., all possess pathway-independent roles in many cellular processes (Table 1.2).

The role of FA signaling in response to DNA damage and genome instability are likely to go hand in hand to the accelerated aging phenotype in FA patients. However, it is also suggested that the FA proteins are involved in both protecting and regulating telomere length [64]. This demonstrates the vast capacity of FA signaling, as FA cells have also been characterize to contain impaired telomeres [65]. Similarly, the FA pathway can regulate the cell cycle (discussed later in detail) either by responding to replicative stress, or through promoting mitotic function [66]. Distinct contrasting roles of FA signaling have also been observed in metabolism [40, 50], where the FA proteins are directly involved in mitochondria function [67, 68], as well as protecting cells from aldehydes [69]. As the former would stimulate non-canonical FA signaling and the latter would appears to activate the canonical FA pathway more favorably in response to the DNA damaging and carcinogenic properties [69-71].

FA signaling acts in a cell context-dependent manner, with the net cellular effects derived from the FA pathway and FA pathway-free functions [50]. Conversely, in the event that the FA pathway is inactivated, a new FA signaling function has been reported [72], rather than many of the clinical issues discussed above.

FA Proteins	Involved in DNA damage	Involved in other cellular processes
FANCA		CD40 signaling pathway; cell proliferation; inflammatory response; T cell differentiation; Sequence-specific DNA binding transcription factor activity
FANCB	DNA damage repair (not entirely dependent on the FA core complex)	
FANCC	TP53 Regulation of DNA Repair Genes	Generic transcription pathway; Gene expression; Diabetes
FANCD1	DNA damage repair, (not entirely dependent on the monoubiquitinated D2/I)	Cell cycle regulation; meiotic recombination; Presynaptic phase of homologous DNA pairing and strand exchange; Resolution of D-loop structures
FANCD2	The HHR6 signaling pathway; The ATM signaling pathway; DNA damage repair; TP53	Replication: Replication-origin firing, Stalled replication forks; Mitochondria function; gene expression;

	regulation of DNA Repair Genes; MiRNA regulation of DDR	
FANCE/F/G	DNA damage repair (not entirely depending on the FA core complex)	
FANCI	The ATR signaling pathway; TP53 regulation of DNA Repair Genes; DNA damage repair	Gene expression
FANCJ	DNA damage repair (not entirely dependent on the monoubiquitinated D2/I); G2/M DNA damage checkpoint	Cell cycle regulation; Cytosolic iron-sulfur cluster assembly; P53 activity; Presynaptic phase of homologous DNA pairing and strand exchange; Resolution of D-loop structures
FANCL	DNA damage repair (not entirely dependent on the FA core complex)	Ubiquitin mediated proteolysis
FANCM	ATR regulator, or a major sensor of the DDR	Stalled replication forks
FANCN	DNA damage repair (HR) (not entirely dependent on monoubiquitinated FANCD2)	Resolution of D-loop structures; Homologous DNA Pairing and Strand Exchange
FANCO	DNA damage repair (not entirely dependent on monoubiquitinated FANCD2)	Meiosis; Resolution of D-loop structures; Megakaryocyte development and platelet production; Cell cycle
FANCP	DNA damage repair (not entirely dependent on monoubiquitinated FANCD2/I)	Resolution of D-loop structures
FANCQ	DNA damage repair (DSB, NER) (not entirely dependent on monoubiquitinated FANCD2)	Transcription
FANCR	DNA damage repair (HR) (not entirely dependent on monoubiquitinated FANCD2/I); ATM signaling	Cell cycle; Meiosis; Rac1/Pak1/p38/MMP-2 pathway
FANCS	DNA damage repair (HR) (not entirely dependent on monoubiquitinated FANCD2/I); ATM signaling	Transcription (ATF-2, E2F, FOXA1 transcription factor networks); Androgen receptor signaling pathway; Aurora A signaling; Cell Cycle Checkpoints; Deubiquitinating; Gene regulation
FANCT	DNA damage repair (not entirely dependent on monoubiquitinated FANCD2/I);	Post-translational protein modification

FANCU	DNA damage repair (not entirely dependent on monoubiquitinated FANCD2/I)	Resolution of D-loop structures; Presynaptic phase of homologous DNA pairing and strand exchange
FANCV	TLS performed by POL1, POLK, REV1 or Zeta; post replication repair (not entirely dependent on monoubiquitinated FANCD2/I)	Cell cycle regulation; Shigellosis; Oocyte meiosis; Endoderm Differentiation
FANCW	Ubiquitination of RPA (not entirely for the activation of the FA pathway)	Ubiquitination; Mediation of p53 ubiquitination for its stability

Table 1.2. The FA pathway-independent roles played by the FA proteins [50].

1.6 Fanconi Anemia Complementation Group D2

FANCD2 is the most evolutionarily conserved FA gene from lower eukaryotes to mammals [73], and shares approximately 20% similarity with invertebrates [6]. A key player within the FA pathway, FANCD2 also serve as important scaffold proteins to promote distinctive enzymatic activities [50]. FANCD2 is a 1,451 amino acid protein present in a non-ubiquitinated (155 kDa) and monoubiquitinated isoform (162 kDA) at basal (Table 1.1). Following activation of the FA pathway, indicated by the expression of monoubiquitination, the ratio of non-ubiquitinated to ubiquitinated FANCD2 shifts towards to the ubiquitinated form (Figure 2.1 A). In contrast to poly-ubiquitination which tags a protein for proteasome degradation [74], FANCD2 monoubiquitination promotes translocation, and is required for chromatin loading (Figure 2.1 B) [75]. Although incidence of FA patients, resulting from mutations in FANCD2 is relatively low, FANCD2 is considered a key central player in the FA signaling pathway, with over 80% of all FA mutations rendering the inability to monoubiquitinate FANCD2 (Table 1.1). Interestingly, in its non-ubiquitinated form, FANCD2 is also known to function via complex formation with BRCA1, FANCG and XRCC3 [76]. This demonstrates the elasticity of FANCD2 to perform distinctive regulatory roles.

Regulation of FANCD2: FANCD2-V1 & FANCD2-V2

Recently, a new study analyzing the structure of FANCD2 concluded that FANCD2 exists in two variants, which present distinctive structural and functional characteristics [77]. The new FANCD2 variant was named FANCD2-V2, in contrast to the long known FANCD2 variant, named FANCD2-V1 for comparison. With this discovery, the FANCD2-V2 variant challenges the longstanding and thoroughly explored variant of FANCD2-V1, which until now has been acknowledged to be the only variant to exist. Due to structural and sequence similarity to FANCD2-V1, FANCD2-V2 was previously overlooked, differing by only 40 of 1471 AAs. Although it is still unclear the precise roles each variant undertakes, the ratio of FANCD2-V2/FANCD2-V1 expression is relatively higher in non-malignant cells/tissues and low stage tumors compared to their malignant counterparts. FANCD2-V2 thus appears to be more inclined to prohibiting the initiation of neoplastic transformation via its more potent tumor suppressive roles [77]. Therefore, transcriptional regulation (polyadenylation) of FANCD2 can have significant impact upon function, even with subtleties in structure.

Regulation of FANCD2 monoubiquitination

As we have already discussed, although it is evolutionarily conserved, FANCD2 function is dependent upon and subject to a number of regulatory mechanisms. In addition to polyadenylation, another transcriptional regulatory function, alternative splicing, has also been shown to significantly influence the activity of FANCD2 [78, 79]. Rather than directly impacting FANCD2 function, alternative splicing of FANCL creates the novel splice variant, FAVL [78, 79]. FAVL expression is heightened in malignancy and impairs FANCD2 monoubiquitination [78, 79]. This is achieved by sequestering FANCL into the cytoplasm for degradation, therefore, FANCL is unable to act as the catalytic unit to monoubiquitinate FANCD2. In our lab, cells with ectopically expressed FAVL to inactivate FANCD2 [79]. Similarly, mutant type (mt) cells carry a point mutation at Lys561 of FANCD2 *in vitro*, which also demonstrates impaired FANCD2 monoubiquitination, compared to their wild type (wt) counterparts.

1.7 FA and Cancer

Numerous studies have long indicated that the FA genes play regulatory roles extending beyond the protection from FA. Mutations in the FA genes have been of great interest in cancer biology, as individuals who carry these mutations inherently possess a much greater susceptibility to cancer [80]. Primarily, the FA genes function to protect individuals from cancer by effectively repairing damage DNA, as well as through other regulatory mechanisms, discussed later. However, studies show that in the absence of DNA damage, FA signaling can also function to promote replication fork stability [81, 82]. From the perspective that cancer is a genetic disease, it is believed to be caused by a series of mutations occurring in both germline and somatic cells [83-85]. Therefore, it comes as no surprise that mutations in the FA and FA associated genes lead to a compromised signaling, which is highly correlated to genome instability and tumorigenesis. [12].

Individuals who suffer from FA are prone to a number of different cancers such as acute myelogenous leukemia, breast cancer, squamous carcinoma of the head and neck, and cancers of gynecological system, skin, esophagus, liver and kidney [40]. Because of this, the median lifespan of FA patients range from 20-30 years [86]. In addition, as FA requires homozygous mutations, individuals who possess heterozygous mutations in any of the FA genes (with the exception of FANCB and FANCR) do not present the majority of FA symptoms, however, do carry a greater risk of developing cancers [86, 87]. Numerous studies have demonstrated a strong relationship exists between non-FA cancer patients carrying germline FA gene mutations and the development of a subset of human cancers [88-90]. Of particular interest, mutation in the breast cancer susceptibility genes (BRCA-related genes; FANCD1/S/N/J) are now often reported in relation to breast & ovarian cancer predisposition in women [91]. Although mutations in BRCA1/2 (FANCD1/S) carries a considerably lower risk in males for breast cancer [91], a recent study has demonstrated the significance of BRCA2 mutations, as a risk factor for aggressive prostate cancer [92].

Transformation of normal cells into cancer cells entail concerted genetic changes in many genes. Studies of non-FA cancer cell lines and human tissues have demonstrated that impaired FA signaling promotes tumorigenesis [78, 79, 93]. Whereby, the occurrence of mutations in FA genes related to the entire FA pathway has been reported to exist in thousands of non-FA tumor/cancers, even up to 52.8% of analyzed cases in bladder urothelial carcinoma (n=127) [87]. Data from The Cancer Genome Atlas shows that the rate of mutations to the FA pathway in other cancers vary depending upon the cancer (Figure 1.2). Whereas, the human genome project demonstrated that a considerable rate of impairment to the FA signaling pathway (near an approximate mean rate of 30%) was present in non-FA human cancers [87]. Importantly, the analysis of the rate of impairment to the FA pathway has been shown to be significantly and positively correlated to tumor stage [87]. Not to mention, epigenetic changes in the FA genes can also impair FA signaling functions, which increase tumorigenic potential [94, 95].

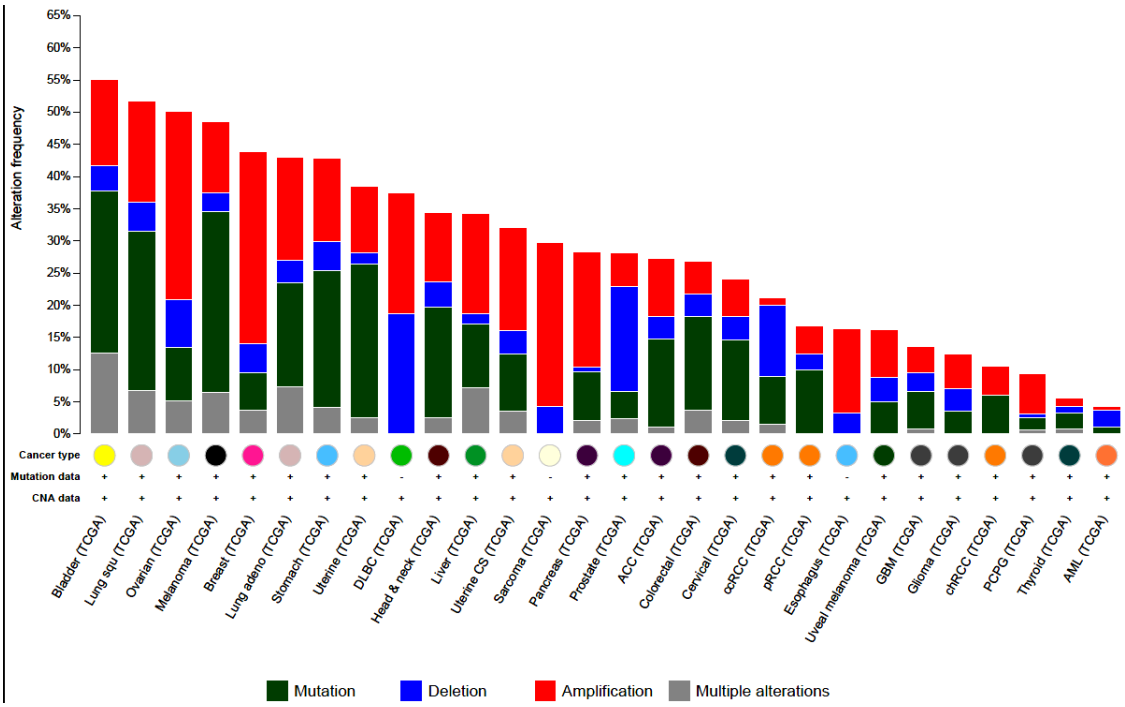


Figure 1.2. The relationship between the mutated FA pathway and human cancer.

As mentioned previously, FA patients are known to have a high incidence of squamous cell carcinoma (43), in which tumor protein p63 (ΔNp63) is highly expressed (48). A recent study has demonstrated that ΔNp63 has been reported to be elevated in FA cells carrying inactivated

FANCD2, but not in FANCD2 null cells or cells carrying wtFANCD2 (49). Therefore, these findings suggests a new role of FA signaling following the inactivation of FANCD2. Whereby, the Gain-of-Function (GOF) phenomenon occurs following the inactivation of FANCD2. This adds a novel layer of complexity in our understanding of the roles of the FA signaling pathway in maintaining a variety of normal cellular processes to protect human cells in diseased states.

Often in the context of cancer, DNA damage is depicted as a double-edge sword. On one hand, DNA damage is known to lead to genome instability and cancer. However, on the other hand, many therapeutic plans targeting cancer often rely on a compromised tumor DDR. Therefore, FA signaling in cancer not only protects normal cells from DNA damage but also tumor cells [40].

1.8 Overview of the Cell Cycle

Cell division is an essential biological process in order for tissue growth, development and reproduction. The cell cycle describes a process of cellular division, which results in the formation of two genetically identical daughter cells from a single parent cell. This fundamental biological process is divided into four phases, gap 1 (G_1), synthesis (S), gap 2 (G_2), and mitosis (M). Over these four phases, genetic material must be faithfully duplicated and divided in pursuance of successful proliferation. Consequently, impaired cell cycle functions can result in aneuploidy, which often leads to apoptosis or malignant transformation [96, 97]. Interphases encompass the phases of G_1, S and G_2, by which cells exist in the majority of the time. During G_1, cells increase their protein and organelle content in preparation for DNA replication.

The transition from G_1 to S phase signified the cells commitment to synthesize DNA from 2n to 4n, and activate cellular replication machinery to replicate DNA. Following the successful completion of the S phase, cells enter G_2, where they prepare for M phase. Alternatively, living cells can also enter irreversible senescence or quiescence (G_0), rather than remaining in the cell cycle. The M phase of the cell cycle involves a series of stages to execute chromosomal and nuclear division, and cytoplasmic division (cytokinesis). These stages consist of prophase, prometaphase, metaphase, anaphase, and telophase and occur in sequential order, with the exception of cytokinesis, which is initiated during anaphase (Figure 1.3).

Initially (prophase), the chromosome condenses and shortens, following mitotic spindle assembly. Mitotic spindles are composed of microtubules (MTs), MT-associated proteins and motor proteins, which are organized by the centrosome, and required to ensure accurate chromosome segregation [98]. During early mitosis (prometaphase) the nuclear envelope breaks down, which allows the MTs to attach to the chromosomes via the kinetochore [98]. These events are regulated by the cytoskeletal motor proteins dynein and kinesin, which are responsible for intracellular cargo transport towards the minus and plus ends of the microtubule, respectively [99]. Following this, the chromosomes align at the equator and kinetochore MTs attach sister chromatids to the opposite poles (metaphase), which are then pulled and separated (anaphase). The nuclear envelope then re-assembles around the two sets of chromosomes, this occurs upon arrival to the spindle poles and centrosome (telophase). Simultaneously, during anaphase and telophase, cytokinesis occurs to ensure two daughter cells with a single nucleus are created.

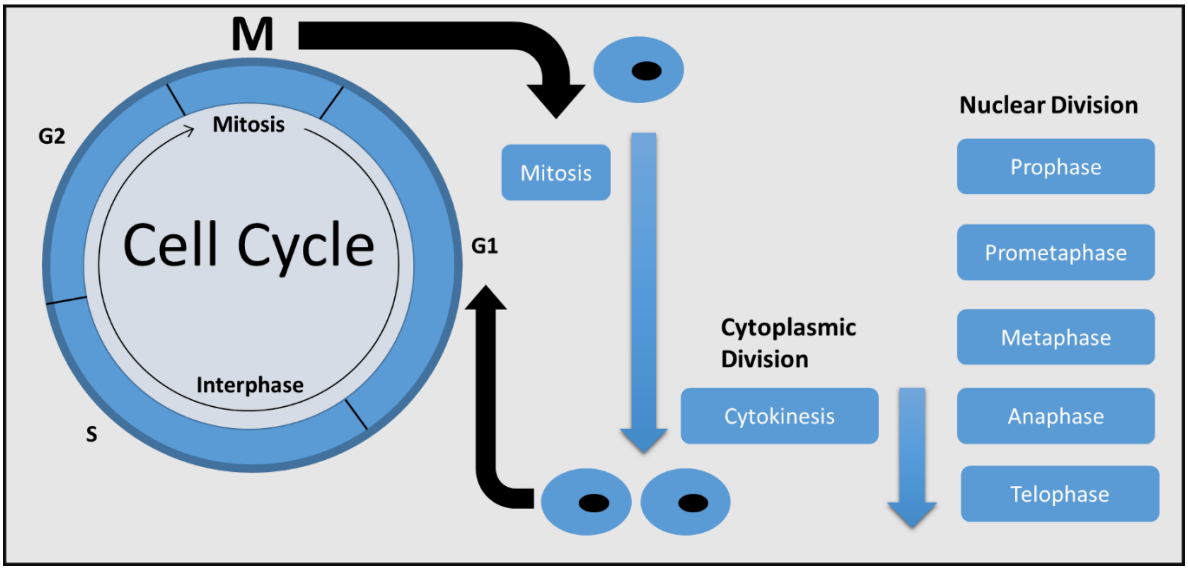


Figure 1.3. Overview of the cell cycle and the phases of mitosis, and cytokinesis

1.9 FA and the Cell Cycle

Studies have reported dysregulation of the cell cycle in FA cells [100]. In addition, FA cells have high incidences of aneuploidy and micronucleation, often occurring as a result of chromosome missegregation [101]. In normal cells, the FA pathway is activated during the S phase of the cell cycle to respond to replicative stress [102]. In addition to genome instability [103], genetic models inhibiting FANCD2 monoubiquitination have also demonstrated deregulated cell proliferation/growth [104]. Following the impairment of FA signaling, the mechanistic consequences extend past deregulation in the DDR and aberrant replication. Indeed, the emerging roles of FA signaling may even encompass the M phase of the cell cycle [105-109].

S phase and Replicative Stress

FA signaling has recently also been acknowledged to play many regulatory roles during DNA replication, in which the entirety of cellular DNA must be faithfully duplicated to maintain genome stability. Furthermore, FA signaling is active in both non-stressed cells at the beginning of DNA synthesis and cells suffering from replicative stress during DNA synthesis [110-113]. To expand upon this, monoubiquitinated FANCD2 has been shown to be capable of functioning with initiators of replication to maintain a proper rate of DNA replication origin firing [111, 112]. *In vitro,* normal cells undergoing the loss of FANCD2 monoubiquitination can lead to a slow rate of replication origin firing, chromosomal abnormality and cellular aging, all hallmarks of cellular deregulation in patients with FA [111].

M phase

During mitosis, the FA pathway is highly regulated via the degradation of FANCM, which not only reduces FANCD2 monoubiquitination [105], but also suggests further regulatory roles of FA signaling within the cell cycle. These studies appear to suggest that replicative roles of FA

signaling are unique to individual proteins within the FA pathway rather than a regulatory function of the whole pathway.

During M phase, FA signaling is highly regulated via the degradation of FANCM. This occurrence not only reduces FANCD2 monoubiquitination but also implements further regulatory roles within the cell cycle [105]. In collaboration with BLM, FA signaling can promote proper chromosomal segregation at fragile sites [106, 107]. Additionally, FANCD2 has been found to be essential for the protection of chromosomal integrity [108]. To achieve this, FANCD2 acts in concert with FANCI and BLM to survive mitosis with acentric chromosomes in a DDR-independent manner [108]. Furthermore, FANCP has been reported to interact with Mus81 and others to promote appropriate chromosome segregation and to avoid mitotic catastrophe [109]. Moreover, crosstalk between FA signaling and other players expand the role of FA signaling in safeguarding chromosome stability during mitosis. In addition, the regulation of FA signaling by p21 (a cyclin-dependent kinase inhibitor) [114] and p53 [115] further supports the role of FA signaling in the regulation of cell proliferation. This possibly extends to all phases of the cell cycle, beyond the phases discussed. However, further research is required to validate this aspect of FA signaling, as it currently remains unclear.

1.10 Nuclear Distribution Protein C

The nuclear distribution protein C (NUDC) was originally identified in the filamentous fungus *Aspergillus nidulans* as a nuclear movement gene in the asexual reproductive cycle [116]. The 42 kDa protein, NUDC, is highly conserved from fungi to human [117] and is essential for cell viability [118, 119], which eludes to its importance in cellular function. Mammalian homologues of the NUDC proteins include NudCL [120], and NudCL2 [121, 122], which all contain a p23 domain that is capable of binding with heat shock protein 90 (Hsp90) [123]. The nuclear distribution (*nud*) genes (Lis1/NUDF, NUDE, NUDA, NUDG and NUDK) encode components and regulators of the dynein/dynactin motor complex [123, 124]. Furthermore, NUDC is characterized as a stable ubiquitous protein, especially abundant in proliferative cells/tissues [125]. Although several

NUDC functions and interacting partners have been identified, there is still a great deal of unknown regarding this important gene/protein.

NUDC in the M phase

The exact role of NUDC in the M phase of the cell cycle is still somewhat unclear, however, NUDC protein expression has been detected to double during M phase [126]. Previous studies have evidenced NUDC in key events across multiple stages of mitosis, and cytokinesis [127]. In addition, NUDC has been observed to be localize at various locations during M phase (Table 1.3). The functions of NUDC through M phase is highly dependent upon protein interaction, complex formation and post-translational modifications. As such, NUDC localization to the mitotic spindle is critical for mitotic progression and requires interaction with echinoderm microtubule-associated protein like 4 (EML4) [128].

Although, understanding the impact of each role requires further investigation, NUDC and other *nud* proteins have been highly implicated in the regulation of dynein activity [120, 121, 127]. As previously mentioned, dynein is a cytoskeletal motor protein involved in chromosomal movement and spindle formation during M phase. In mammalian cells, NUDC interacts with Lis1/NUDF [129], kinesin-1 and dynein/dynactin to promote anterograde and retrograde transport along the MT [130]. Furthermore, NudCL, which shares similar sequence homology with NUDC, is suggested to promote dynein stability [120].

Phase of Mitosis	NUDC Subcellular Localization
Prophase	MTOC
Prometaphase	MTOC, mitotic spindles
Metaphase	MTOC, mitotic spindles
Anaphase	Midzone of MTs
Telophase	Midzone of MTs
Cytokinesis	Midbody

Table 1.3. NUDC localization during M phase [127, 131, 132]. Abbreviations: MTOC, microtubule organizing center; MTs, microtubules.

Other than its reported roles concerning dynein interaction, NUDC has also been observed to interact with Polo-like Kinase 1 (Plk1) independent of the dynein/dynactin complex [133]. Plk1 is essential in mitotic progression and performs multiple regulatory roles throughout the distinct phases of mitosis and cytokinesis [134, 135]. Studies have characterized that Plk1 phosphorylates NUDC at Ser274 and Ser326 [133]. Following Plk1 mediated phosphorylation, NUDC directs Plk1 translocation to the outer plate of the kinetochore (Figure 1.4) [136]. In addition, this process is required for correct chromosomal alignment at the metaphase plate, chromosomal congression via Centromere Protein E (CENP-E) during prometaphase (Figure 1.4), and promotes kinetochore-MT attachment [136].

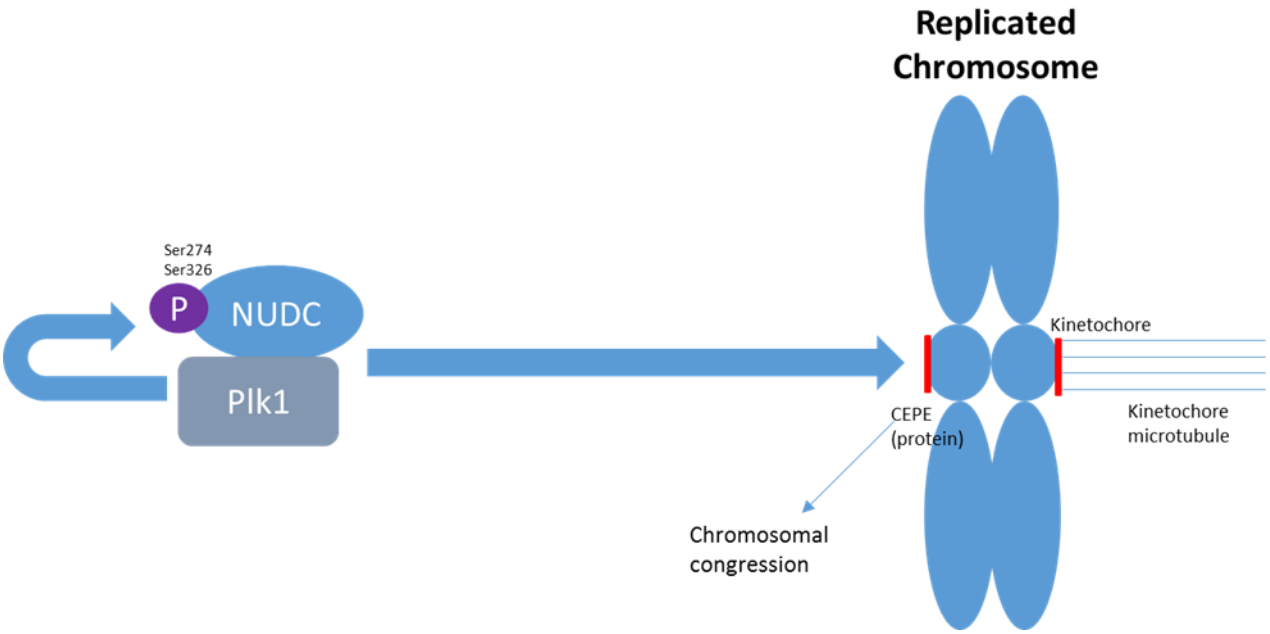


Figure 1.4. Plk1 phosphorylates NUDC in M phase. Plk1 phosphorylation of NUDC leads to co-localization of the Plk1-NUDC complex to the outer plate of the kinetochore.

Post-translational modifications of mitotic proteins are known to regulate and drive M phase forward, with NUDC being no exception [137]. In addition to Plk1 [133, 136], Aurora B, a serine/threonine protein kinase implicated in chromosome condensation, segregation, and cell division [138, 139] has also been previously reported to phosphorylate NUDC [131].

Immunoprecipitation (IP) experiments revealed that Aurora B interacts with NUDC at both the early and late phases of mitosis [131]. However, Aurora B mediated phosphorylation of NUDC at Thr40 occurs during cytokinesis to regulate intercellular bridges and cell abscission [131]. Cyclin-dependent kinase 1 (Cdk-1) has also been suggested to phosphorylate NUDC [131], although, experimental data is currently lacking.

NUDC deacetylation by histone deacetylase 3 (HDAC3) has also been reported to regulate M phase [132, 137]. NUDC acetylation occurs on Lys39, however, without deacetylation during M phase, chromosome alignment, segregation, and spindle formation is impaired [132]. Thus, NUDC has been observed to co-localize with HDAC3 during prometaphase and metaphase on the mitotic spindles [132]. Furthermore, NUDC deacetylation is also suggested to contribute to promote M phase progression [132].

NUDC and tumorigenesis

Mitosis is a heavily regulated process that requires precise coordination of molecular signals and events, in order to avoid genetic inaccuracies, which can lead to genome instability. Previously, NUDC has implicated in tumorigenesis and cancer, although the mechanism behind this is still unknown [123]. The rate of mutations in NUDC has been identified to be relatively low (below 10% in various cancers) (Figure 1.5), this indicates NUDC functions may be impaired tumorigenesis irrelevant to gene mutations. In tumor and leukemia cells, NUDC is upregulated [140-142], however, overexpression of NUDC has shown to inhibit tumorigenesis of prostate cancer cells [141]. This phenomenon is suggested to be due to failures in cell division, as NUDC overexpression leads to cells with enlarged nucleuses, as well as bi-nucleated and multi-nucleated cells (Figure 1.6) [141]. Similarly, these findings are consistent with other studies reporting NUDC overexpression reducing proliferation, and promoting aneuploidy and polyploidy [127].

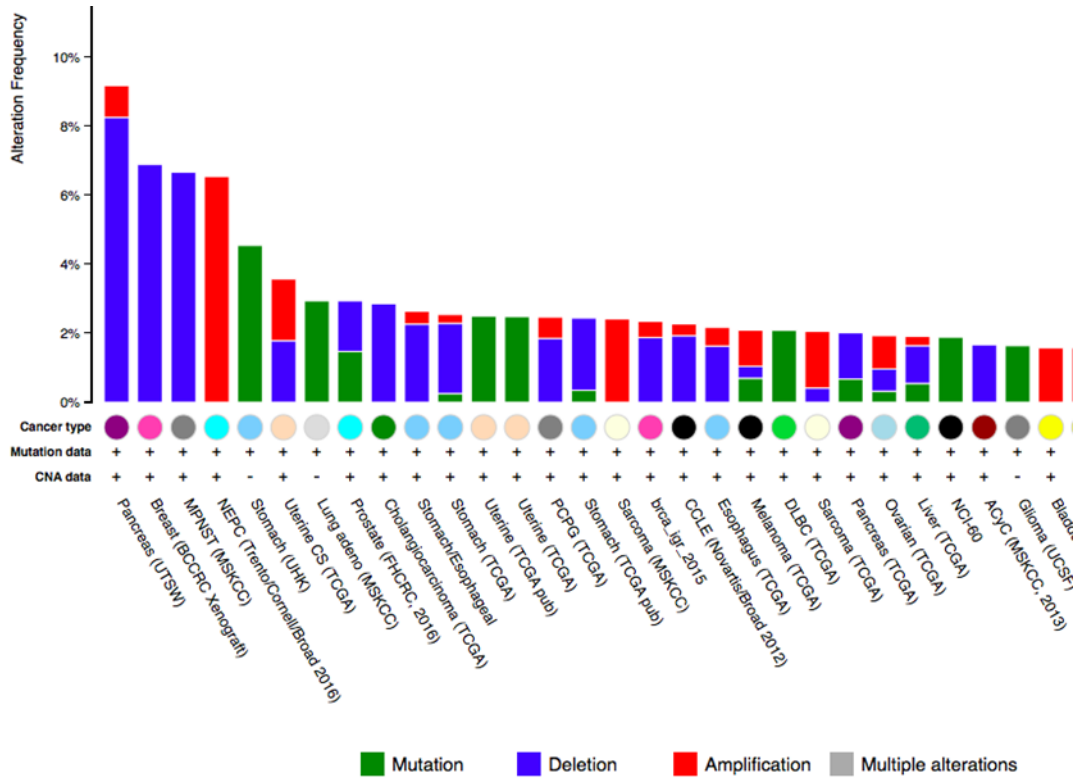


Figure 1.5. The incidence of NUDC mutations in human cancer. Relatively low in various cancers, however, NUDC is subject to a number of post-transcriptional modifications, which may implicate NUDC function in tumorigenesis.

Conversely, studies that downregulate or knockout NUDC have reported the mislocalization of dynein related proteins, impaired dynein functions, cell growth inhibition, lethality and/or multiple mitotic defects such as impaired kinetochore-microtubule attachment, elongated intercellular bridges, impaired spindle formation, chromosome miscongression, and more [120, 130-133]. Aberrant NUDC expression, both overexpression and downregulation/knockout are shown to lead to cytokinesis failure and G2/M block, where cells exiting the S phase are unable to correctly divide (Figure 1.6) [143]. Therefore, NUDC may act similarly as a proto-oncogene to promote regular M phase functions. Further investigation is required to understand the evolutionarily conserved roles of NUDC in M phase and its relationship with tumorigenesis. As

mentioned above, NUDC function is not only dependent upon its expression, but also protein interaction(s) and posttranslational modifications.

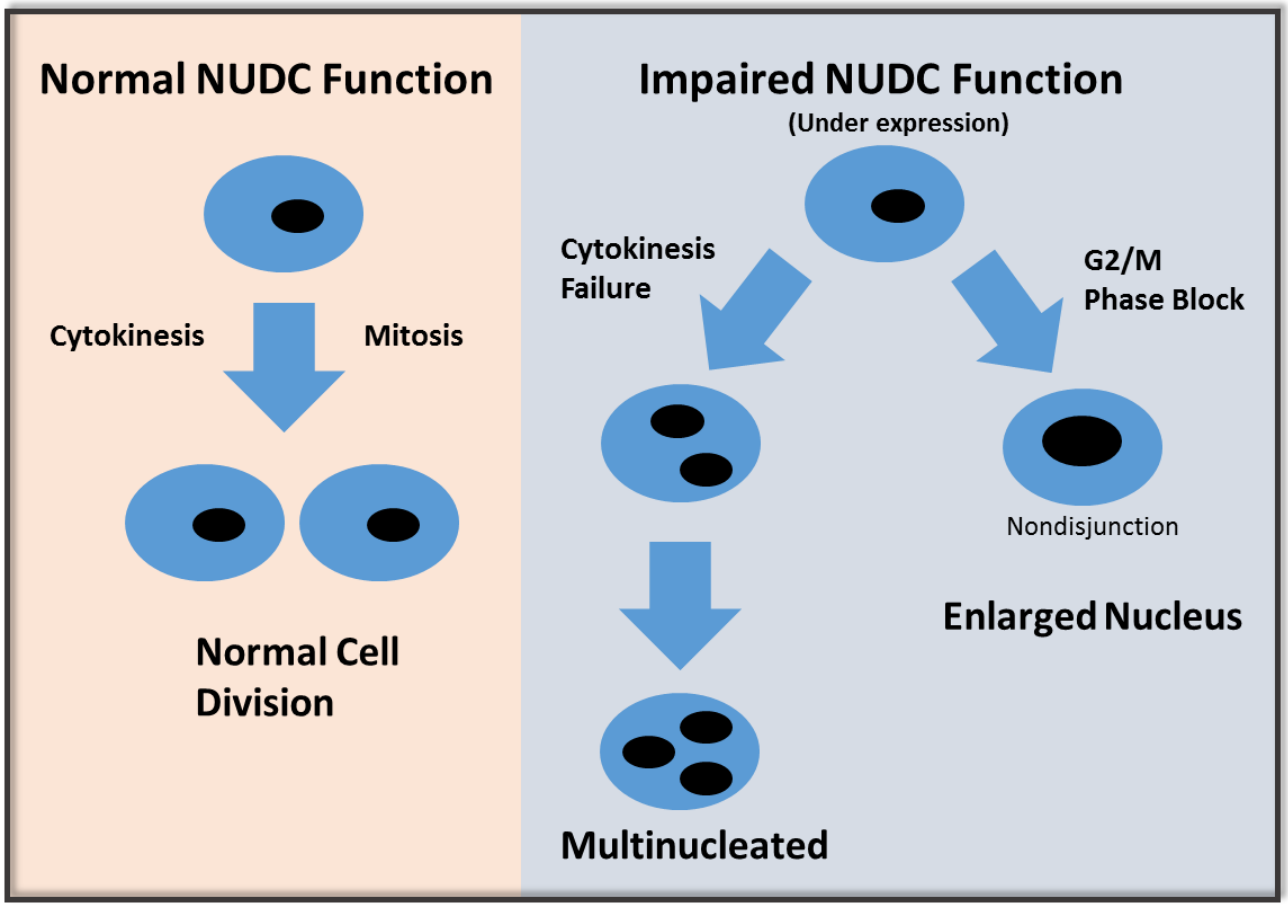


Fig 1.6. The role of NUDC in cell division, adapted from [143]. NUDC and its role in cell division; NUDC is an important regulatory protein during cell division. When NUDC function is impaired, this leads to events such as failure in cytokinesis and checkpoint inactivation of the G2/M phase block leading to aneuploidy/polyploidy, and senescence, apoptosis.

1.11 Aims of the Thesis

The role of FA signaling in response to DNA damage and replicative stress have been subject to a great deal of investigation and is well characterized. However, the role of FA signaling in regards to the regulation of the cell cycle, specifically M phase, have yet to be elucidated. In recent years, FA signaling has clearly demonstrated its ability to regulate numerous biological functions outside of the DDR. With the emergence of new FA signaling functions independent of the FA pathway, non-canonical FA signaling demonstrates great potential to uncover new roles of the FA genes. Furthermore, new understanding of the vast regulatory roles of FA signaling suggests that the FA proteins would also interact with previously unknown targets.

Currently, with respect to the literature, the focus upon FA signaling is primarily related to its role in the DDR and replicative stress. However, researchers have exposed that FA signaling is involved in processes extending outside of the DDR, as well as all phase of the cell cycle. Therefore, the goal of this thesis is to investigate the role of FA signaling in relation to the M phase of the cell cycle. In addition, we aim to characterize the relationship between FANCD2 and NUDC, and validate their roles as M phase regulatory proteins.

Chapter Two: Manuscript

2.1 Abstract

Fanconi anemia (FA) is a genetic disease resulting from germline mutations in any of the 22 FA genes. Recently, evidence has demonstrated that FA signaling regulates a vast number of biological processes including the cell cycle. However, current research regarding FA signaling and the cell cycle mainly revolves around the role of FA during S phase. Despite this, we believe that FA signaling governs all phases of the cell cycle, with individual FA proteins performing independent functions. Here, we report that FA complementation group D2 (FANCD2) interacts with the M-phase protein, nuclear distribution protein C (NUDC). Using MS/MS mass spectrometry analysis we identified a number of proteins between 38-52 kDa, which interact with FANCD2. In addition, through gel filtration, immunoprecipitation (IP) and immunofluorescence (IF) analysis, we validated this relationship between FANCD2-NUDC interaction. Furthermore, we demonstrated that FANCD2 plays an important role in chromosome segregation, which may be achieve via its interaction with NUDC.

2.2 Introduction

Fanconi anemia (FA) is a rare genetic disease characterized by a number of congenital defects, bone marrow failure and an increased susceptibility to hematological and non-hematological malignancies [2]. To date, 22 FA genes (FANCA /B /C /D1 /D2 /E /F /G /I /J /L /M /N /O /P /Q /R /S /T /U /V & /W) have been identified, which comprises a vast signaling network that includes the FA-BRCA pathway as well as a number of non-canonical FA pathways [144]. Impairment to any of the FA genes/proteins have demonstrated detrimental effects that lead to an increased rate of gene mutations, aging and cancer [40, 145, 146]. Previously, FA signaling has been primarily characterized as an essential component in the repair of DNA damage, especially in interstrand crosslinks (ICLs) [5]. Although this still holds true, the discovery of many novel interacting partner has prompted an expanded understanding regarding the regulatory functions of FA signaling. In recent years, FA signaling has demonstrated that it performs roles related to DNA replication [111, 112], the cell cycle [100], mitochondrial function [68], and metabolism [104]. Similarly, many FA signaling functions are carried out to maintain genome stability and/or prevent tumorigenesis [40, 145, 147].

In the event of DNA damage or replicative stress the FA signaling pathway is activated [4]. Activation of the FA pathway is characterized by FANCD2 and FANCI monoubiquitination, which signals to the downstream functional units [6]. In addition, FANCD2 is the most evolutionarily conserved FA gene, and arguably the most important player within the FA pathway [148]. Recently, using MS/MS mass spectrometry we identified a number of interacting proteins between 38-52 kDa and FANCD2. Of interest, we identified nuclear distribution protein C (NUDC) at 42 kDa. NUDC is associated with the microtubule motor dynein/dynactin complex, and promotes the regulation of M phase [123, 124].

NUDC is a highly conserved protein from fungi to humans and belongs to the nuclear distribution (*nud*) gene family. In fungi, NUDC is required for nuclear distribution for asexual reproduction [116], and is also essential to cell viability [119]. Whereas in humans, NUDC functions to regulate mitosis and cytokinesis throughout various cellular sub-compartments and numerous interacting

proteins [127]. Additionally, NUDC is also prone to a number of post-translational modifications such as acetylation and phosphorylation to regulate mitosis and cytokinesis [133, 137]. Studies which have investigated impaired NUDC function, have reported defects in M phase, such as cytokinesis failure that led to micronucleation, and G2/M phase block, leading to nondisjunction [127, 141]. Furthermore, overexpression of NUDC has been shown to inhibit tumorigenesis in prostate cancer cells [141]. Despite this, the mechanism by which NUDC regulates M phase is still unclear.

FA cells exhibit a high incidence of aneuploidy and micronucleation, with a high rate of bi- and multinucleated cells, chromosomal breaks at fragile sites, and DNA bridges during mitosis [100, 149]. Research has implicated FANCD2 in genome surveillance, acting as a checkpoint mechanism, whereby irradiated FA fibroblasts enter into abnormal mitosis [150]. Previously, we identified a role of FANCD2 in chromosomal segregation (unpublished data). Whereas more recently, we demonstrated that inhibition of FANCD2 monoubiquitination via a reduction in FANCL activity influenced the proliferative rate of cells *in vitro* [79]. Therefore, we believe FA signaling governs all phases of the cell cycle, not only during replicative stress [144]. Similarly, the level of NUDC expression also correlates to the cell proliferation and chromosomal segregation in cells *in vitro* [141].

This study investigates the role of FANCD2 during M phase using a previously established cell system, involving FANCL knockdown leading to slow, medium and fast growing cells [79]. Here, we validated the proteomic interaction between FANCD2 and NUDC, and aimed to identify a role for FANCD2 in M phase to account for our previous observations *in vitro*. These studies for the first time suggest that abnormalities triggered by impaired FA signaling may be attributed to the loss of a functional link between FANCD2 and NUDC.

2.3 Materials and Methods

Cell culture

Colonic epithelial cells (CRL-1790), human embryonic kidney 293T cells, human bone Osteosarcoma Epithelial Cells (U2OS) cells, and human ovarian adenocarcinoma line (IGROV1) cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were grown in high glucose Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and 0.5% pen-strep. Cells were cultured in a humidified incubator contain 5% CO_2 at 37^oC, and subculture appropriately. As previously described [79], the slow, medium and fast cell system is derived from cells following FANCL knockdown.

Antibodies and Chemicals

NUDC, vinculin, RFP and IgG antibodies were purchased from Santa Cruz (Dallas, TX, USA). FANCD2 antibodies were obtained from NOVUS (Littleton, CO, USA). Flag and β-actin antibodies as well as mitomycin C (MMC), cisplatin, crystal violet solution, puromycin, blue dextran, and molecular weight markers used for gel filtration were purchased from Sigma (St. Louis, MO, USA).

DNA Damage Interventions

In this study, in order to induce DNA damage, cells were subject to various DNA damaging agents such as ultraviolet C (UVC), cisplatin, MMC and irradiation (IR). IGROV1 cells were treated with 50 j/m^2 UVC and harvested at 30 min and 2 h post treatment. The CRL-1790 cells were treated with 50 ng/ml MMC and harvested 11 d post treatment. U2OS cells received 10 Gy IR and were collected at 0, 4, 8, 24 and 36 h post IR. U2OS cells were treated with cisplatin at 4 μM/ml and were harvested at 36 h post treatment.

Protein Fraction Preparation

As previously described [79], lysates were prepared from 293T cells and separated in cytoplasmic, nuclear and chromatin fractions as described in the protocols provided by the manufacturer (Pierce, Thermo Fish Scientific, Waltham, MA).

Western Blot

Lysates in SDS-lysis buffer were pipetted into 5% or 8% Tris-glycine SDS-polyacrylamide gels respectively, and separated via electrophoresis. Subsequently, proteins were transferred onto polyvinylidene fluoride (PVDF) membranes. Ponceau staining was used as quality control, and membranes were washed and blocked in 5% non-fat milk/PBS. Membranes were incubated overnight at 4^{o}C with primary antibody. Following this, membranes were washed with PBST and then incubated with secondary antibody for 1 h at room temperature. Immunoreactive bands were detected via enhanced chemiluminescence using Detection Reagent Luminol Enhancer and Detection Reagent 1 Peroxide Solution mixed in a 1:1 ratio (Pierce, Thermo Fish Scientific, Waltham, MA) and premium X-ray film (Phenix research products, Candler, NC, USA).

Cell Proliferation

As previously described [55], cells were equally plated at day 0. These cells were stained with crystal violet dye and formaldehyde at various time points and washed with PSB. Following a standard washing protocol, images were taken to analyze the cell confluence. Samples were analyzed in triplicates.

Mass Spectroscopy

The 8%-16% gradient SDS-PAGE gel was used to resolve FANCD2 elutes, which were prepared from CRL-1790 vector, slow, medium and fast cells. The protein gel was stained with Coomassie

blue and analyzed by MS/MS mass spectroscopy for peptide fragmentation at the Harvard Medical School Taplin Biological Mass Spectrometry Facility.

Gel Filtration

Gel filtration analysis was performed as previously described [21]. Nuclear extracts were isolated from CLR-1790 vector, slow, medium and fast cells. Cytoplasmic and nuclear fractionations were prepared with the NE-PER Nuclear and Cytoplasmic Extraction Reagent Kit (Pierce, Thermo Fish Scientific, Waltham, MA) as per the manufacturer's instructions. The nuclear extracts were directly applied to a sepharose 6B column (Sigma) equilibrated with column running buffer containing 20 mM HEPES (pH 7.9), 200 mM NaCl, 1 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mg/ml leupeptin, 2 mg/ml aprotinin, 0.1% NP-40 and 5% glycerol. Fractions of 1 ml were collected and analyzed by SDS-PAGE and immunoblotting. 2000 kDa blue dextran and 669 kDa thyroglobulin were used to determine the sizes of the fractions.

TOPO-cloning

Using the NCBI database, primers were designed via the PrimerQuest Tool specifically to target NUDC NM_006600.3, XM_017000094.1, XM_011540529.1, XM_017000095.1, XM_011540530.1 and XM_017000096.1, in conjunction with the alignment software Serial Cloner 2-6 (Table 3.1). The TOPO TA kit was purchased from Invitrogen, and PCR products were obtained as per the manufacturer's instructions. 10% 3M NaOac (pH 5.2) was added to the PCR product, along with isopropanol in a 1:1 ratio of the final concentration. Samples were frozen in -80^{o}C for 10 min, thawed and spun down at 8,000 RPM. Following this, 200 µl 75% ethanol was used to wash, then precipitate DNA. Upon appropriate band detection, the protocol was continued as per manufacturer's instructions. 1-10 ng of DNA was added to the DH5α competent cells, which were then incubated on ice for 30 min followed by 20 s of heat shock at 42^{o}C, then incubated on ice again for 2 min. Following this, 1 ml LB medium was added to the cells and shaken at 225 RPM in

37°C for 1 h. Ampicillin and X-gal was spread onto the agar, followed by DH5α competent cell transformation, whereby, the plate(s) were incubate overnight at 37°C. Using the miniprep/midiprep kit purchased from Qiagen, we extracted plasmid DNA from bacteria as per the manufacturer's protocol, and DNA concentration was quantified via spectrophotometer using the Nanodrop.

	Forward	Reverse
NUDC X1 Primer Sequence	5'- ATG ATC TCA AAC TAC AGA AAT GGC AGT -3'	5'-TCC CAG AGA GTGGGA AAG A -3'
NUDC X2 Primer Sequence	5'- ATG GGC GGA GAG CAG GAG -3'	5'-TCC CAG AGA GTGGGA AAG A -3'

Table 2.1 NUDC X1 and X2 primer sequences

Ligation

NUDC-RFP and pLKO.1-NUDC ligations were performed using T4 ligase, using a 3:1 ratio in molecular concentration. Alkaline phosphatase, calf intestinal (CIP) was applied to samples following plasmid cutting and purification, and samples were incubated for 1 h at 37°C to avoid dephosphorylation. The ligation reaction mixture (according to the protocol) was gently mixed and incubated at 16°C overnight, then heated for 10 min at 65°C. Once inactivated the reaction was chilled on ice and transformed in DH5α competent cells.

Co-Transfection

293T cells were cultured in DMEM containing 10% FBS. Plasmids and siRNA transient transfections were performed using Lipofectamine 3000 (Invitrogen) as per the manufacturer's instructions. Once plasmids were added to the P3000 reagent, eppendorfs were vigorously vortexed and incubated for 5 min. Following this, the contents of the eppendorf was transferred into the eppendorf containing opti-medium and Lipofectamine 3000, which were mildly mixed and incubated for 25-30 min. Cells were washed with 1X PBS, prior to adding the transfection

mix. Following this, 16-24 h post transfection (pending cell condition), the opti-medium and the transfection mixture was replaced with DMEM and incubated at 37^{o}C overnight. Subsequently, cells were split and harvested for analysis.

Immunoprecipitation Assay

Previously described [79], however, both scrapping and trypsinized collection methods were utilized. Cells were harvested and fixed in 37% formaldehyde for 3-5 min, glycine was added to 1 M of the final concentration, and gently rocked for 10-15 min. Subsequently, cells were then washed and lysed. The lysates were incubated with monoclonal anti-flag, and NUDC antibodies, respectively, overnight at 4^{o}C, and then incubation with protein A-sepharose (Invitrogen, Thermo Fisher Scientific, Waltham, MA) for 2 h. A beads were washed with medium IP wash buffer and then boiled in SDS-lysis buffer for 5 min.

Gene knockdown by Lentiviral shRNA and Puromycin Selection

Lentiviral transduction was performed as previously described [55]. A set of pLKO.1 plasmid containing shRNA targeting NUDC (NM_006600.3) (forward oligo; 5'-CCGGAACGATTTGCCCAGCTCCTCTCGAGAGGAGCTGGGCAAATCGTTTTTTTG -3' and reverse oligo; 5'-AATTCAAAAAAACGATTTGCCCAGCTCCTCTCGAGAAGAGGAGCTGGGCAAATCGTT-3') with the pLKO.1 empty vector (EV) was used to generate corresponding lentiviruses. 293T cells were transfected with packaging plasmid psPAX2, envelope pMD2G and hairpin-pLKO.1 vector and incubated at 37^{o}C 5% CO_2. Following an 18 h incubation, media was changed and incubation continued for another 24 h before the lentiviral particles were harvested via a 0.22 filter unit and this was repeated. U2OS cells were then infected at various titers by adding the collected virus to the cells (1/1, 1/2, 1/5, 1/10), and cells were incubated at 37^{o}C overnight. 24 h post infection the media was changed again, which contained 0.5-4 µg/ml puromycin for puromycin selection. Following 1 w post puromycin selection, cells were harvested and western blot (WB) confirmed expression of NUDC.

Fluorescent Activated Cell Sorting (FACS)

Cells were harvested and fixed in 75% ethanol and prepared for FACS analysis using propidium iodide staining (500 µl PI, 200 µl RNase (1 µg/ml), 10 ml 1X PBS) to measure DNA content.

2.4 Results

Monoubiquitination of FANCD2 in response to UVC treatment

From this experiment, we observed that following UVC treatment FANCD2 monoubiquitination increased compared to the control samples (CON) and peaked 30 min post UVC treatment. Conversely, the expression of non-ubiquitinated FANCD2 appeared to be inversely related to the trend seen in the FANCD2-monubiquitinated form (Figure 2.1A). In addition, following UVC treatment, the expression of FANCD2 in the cytoplasm appeared to decrease, and was expressed higher in the chromatin compared to CON (Figure 2.1B).

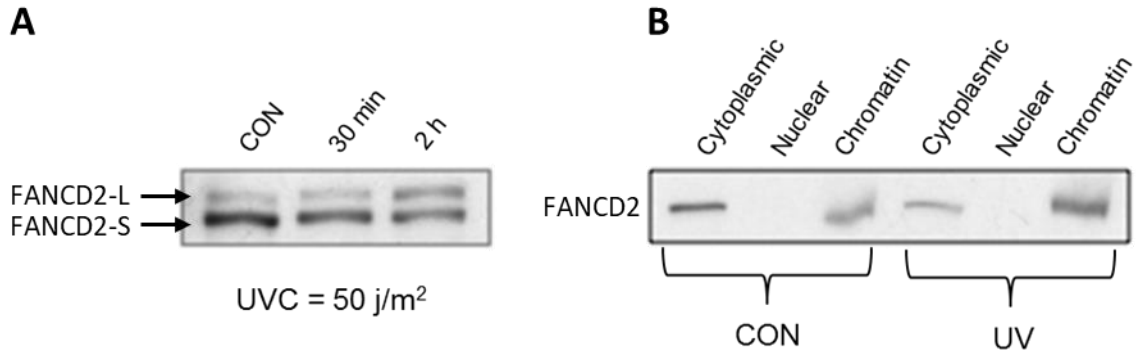


Figure 2.1. Western blot representation of FANCD2 activity. (A) Basal FANCD2 expression compared FANCD2 expression following UVC treatment (50 j/m^2) at time points 30 min and 2 h post treatment. FANCD2-L (top band) represents the monoubiquitinated FANCD2 and FANCD2-S (bottom band) represents non-ubiquitinated FANCD2. (B) Localization of FANCD2 protein at basal compared to 2 h post UVC treatment (50 j/m^2).

Slow, Medium and Fast Cells growth rates

As shown in Fig 2.1 and viewed in [40, 144], FANCD2 is the center of FA signaling. To understand the molecular mechanisms underlying the abnormality triggered by impaired signaling, we decided to use inactivated FANCD2-containing cells to study cell proliferative defects caused by comprised FA signaling. The CLR cell set [79] was derived from reducing FANCL activity to inhibit FANCD2 monoubiquitination (Figure 2.2A). This contains vector (control) cells, FANCL↓ (slow) cells, FANCL↓ cells that have been growing for 2 years (medium) and FANCL↓ cells that have been growing for 3 years (fast). In our preliminary studies, we treated cells with 50 ng/ml Mitomycin C (MMC). Following 11 d post treatment, slow cells were detected at a lower cell count compared to their vector (control) counterparts. Similarly, medium and fast cells exhibit a higher cell count compared to slow cells. Moreover, fast cells exhibit a higher cell count compared to medium cells 11 d post MMC treatment (Figure 2.2B).

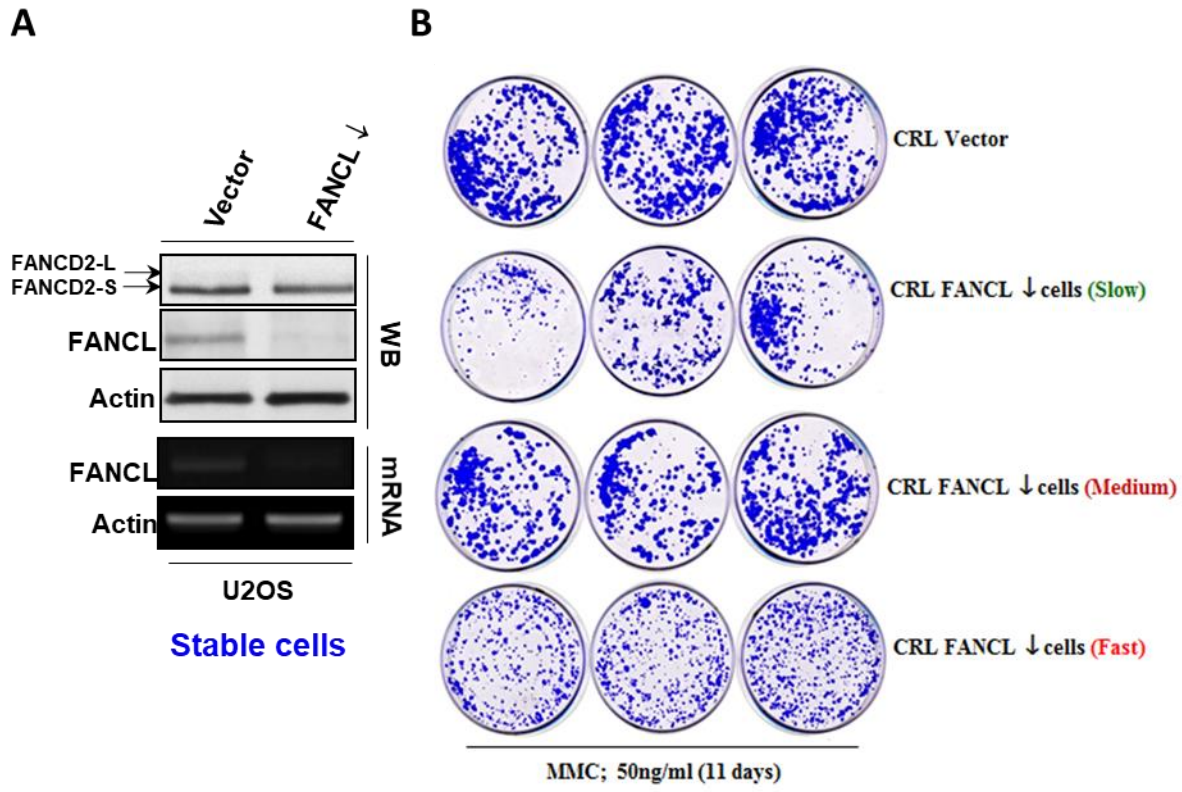


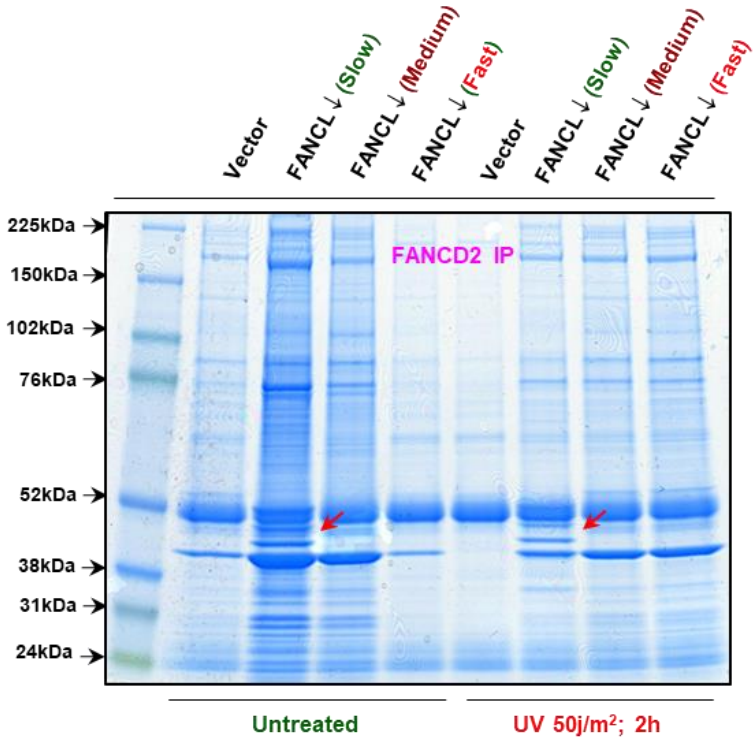
Figure 2.2 FANCL knockdown in CLR-1790 cells. (A) Cell with FANCL knockdown exhibit a reduction in FANCL mRNA and FANCL protein expression. At basal, FANCD2 monoubiquitination

is reduced in FANCL knockdown compared to vector (control) cells. FANCD2-L represent monoubiquitinated FANCD2. FANCD2-S represents non-ubiquitinated FANCD2. (B) The proliferative response to MMC in CRL slow, medium and fast cells following 11 d post MMC treatment.

Identifying FANCD2 and NUDC interaction

Using the CRL cells previously described [79], FANCD2-IP analysis revealed an interaction between FANCD2 and protein(s) between 38-52 kDa, in cells treated with and without UVC treatment (Figure 2.3A). Following this discovery, using a proteomic approach, MS/MS mass spectroscopy identified eukaryotic translation initiation factor 4A1 (EIF4A1), RNA binding motif protein, X-linked (RBMX), 26S protease regulatory subunit 7 (PSMC2), regulator of chromosome condensation 1 (RCC1), nuclear distribution protein (NUDC) and vasodilator-simulated phosphoprotein (VASP) as interacting proteins of FANCD2 (Figure 2.3B).

A



B

Unique	Total	reference	AVG	Gene Symbol	MWT(kDa)
21	30	P60842_IF4A1_HUMAN	3.0092	EIF4A1	46.12
20	43	P62736_ACTA_HUMAN	2.7032	ACTA2	41.98
18	19	P38919_IF4A3_HUMAN	3.1268	EIF4A3	46.84
18	18	Q9UQ80_PA2G4_HUMAN	2.8096	PA2G4	43.76
17	34	P38159_RBMX_HUMAN	2.2891	RBMX	42.31
17	20	P49411_EFTU_HUMAN	2.9507	TUFM	49.51
17	19	P68104_EF1A1_HUMAN	2.8884	EEF1A1	50.11
15	15	P35998_PRS7_HUMAN	2.7649	PSMC2	48.60
13	22	O00303_EIF3F_HUMAN	2.7449	EIF3F	37.54
13	13	P18754_RCC1_HUMAN	2.8718	RCC1	44.94
13	13	Q9Y266_NUDC_HUMAN	2.7982	NUDC	38.22
12	19	P50552_VASP_HUMAN	2.8470	VASP	39.81
12	14	P61158_ARP3_HUMAN	2.7719	ACTR3	47.34
12	12	Q15149_PLEC_HUMAN	2.9869	PLEC	531.47
11	14	Q12905_ILF2_HUMAN	3.1804	ILF2	43.04
11	13	P26641_EF1G_HUMAN	2.7610	EEF1G	50.09
11	12	P60228_EIF3E_HUMAN	2.8738	EIF3E	52.19
10	15	P39023_RL3_HUMAN	2.5142	RPL3	46.08
10	11	P39748_FEN1_HUMAN	2.9966	FEN1	42.57

Figure 2.3 FANCD2 interaction with proteins between 38-52 kDa. (A) SDS-PAGE gels represent FANCD2-IP in CRL slow, medium and fast cells with and without UVC treatment following coomassie blue staining. (B) MS/MS mass spectroscopy identifies a number of 38-52 kDa protein, which interact with FANCD2.

Subsequently, our gel fractionation study demonstrated that the peak expression of NUDC for the CRL vector and CRL slow cells coincided with the same peak as FANCD2 (Figure 2.4)

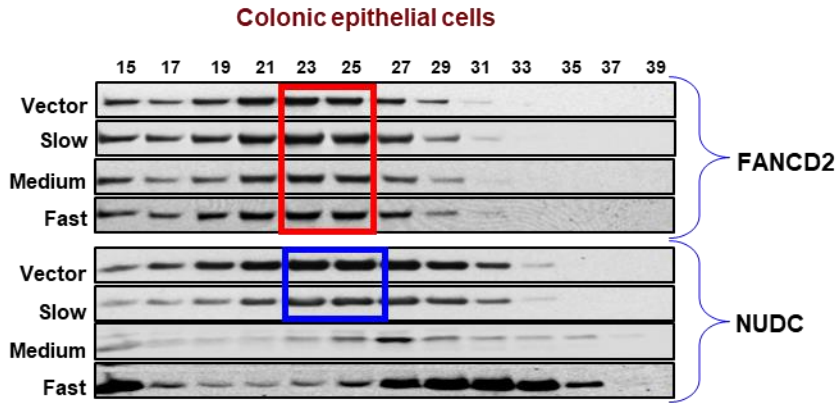


Figure 2.4 FANCD2 and NUDC gel filtration. FANCD2 and NUDC peak at the same gel-filtration fractions prepared from the vector and slow cells but not in medium and fast cells.

Whilst continuing to use the CRL cell set, endogenous immunoprecipitation (IP) experiments demonstrated that FANCD2 interacts with NUDC. However, this interaction appears to be diminished in fast cells, and in medium cells to a lesser extent compared to the vector and slow cells. No differences were observed between the vector and slow cells in FANCD2-NUDC IP (Figure 2.5).

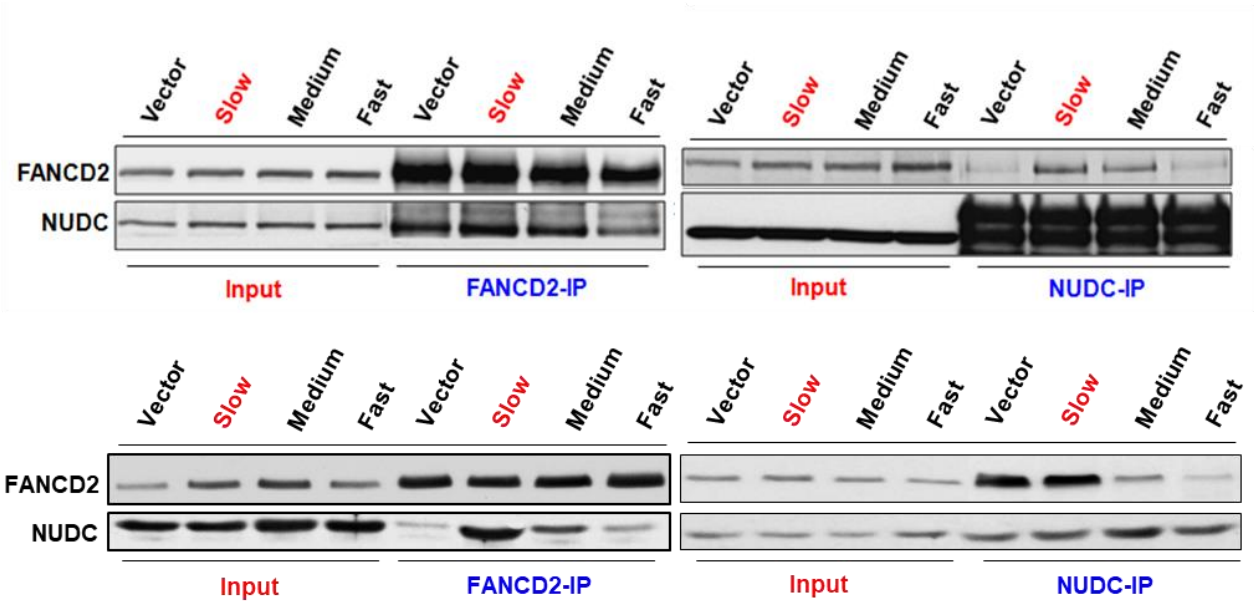


Figure 2.5 Endogenous FANCD2-NUDC IP.

TOPO Cloning NUDC

Next, we aimed to confirm the interaction between FANCD2 and NUDC *in situ.* In order to achieve this, we cloned NUDC using the NCBI databased to designed primers for NUDC X1 and X2 (Table 2.1)(Figure 2.6A). Using these primers, we successfully cloned NUDC (NM_00600.3), NUDC isoform X1 (XM_017000094.1) and NUDC isoform X4 (XM_011540529.1)(Figure 2.6C). Based upon the TOPO-NUDC map (Figure 2.6B), we used EcoRI and compared the detected bands via agarose gel electrophoresis (Figure 2.6C). Although we successfully cloned two novel isoforms of NUDC, we decided to continue our investigation using the NUDC clone most commonly reported in the literature.

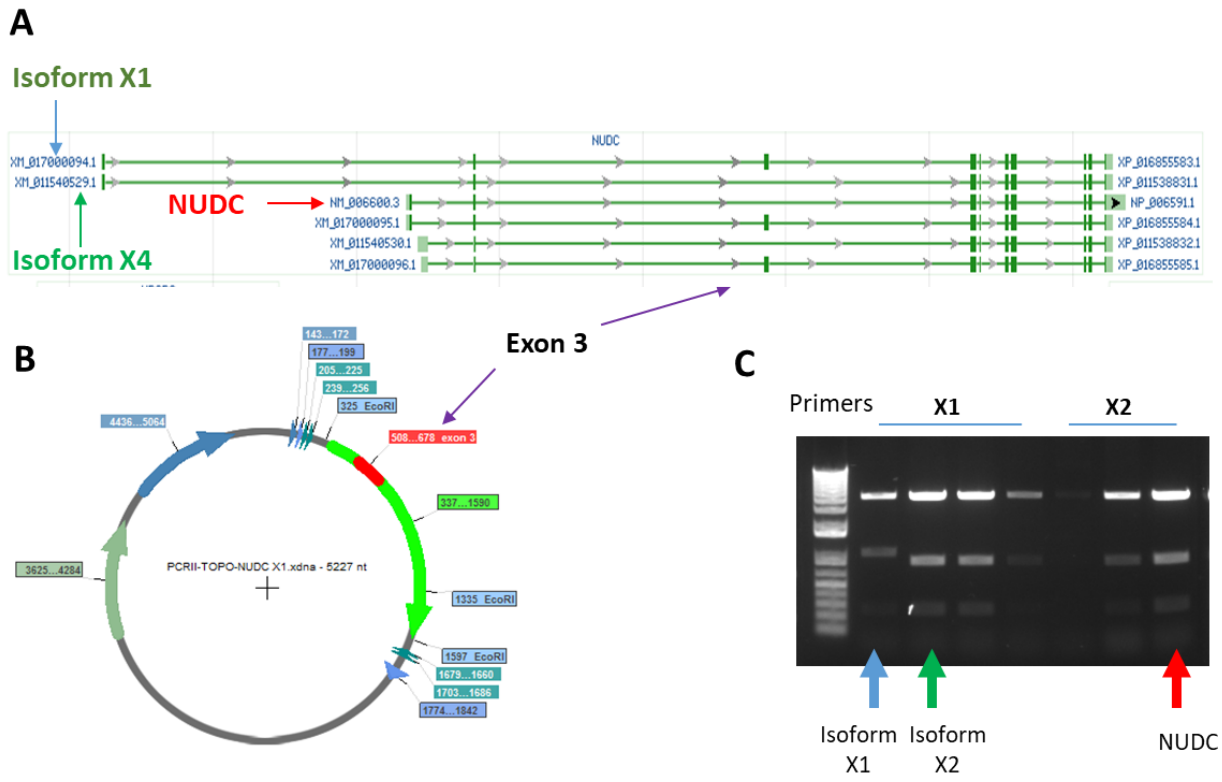


Figure 2.6 TA TOPO cloning and identification of NUDC & NUDC isoforms. (A) Visual representation of the NUDC gene and its variants from NCBI. (B) Map of the TOPO-NUDC construct, which include the respective cutting sites of EcoRI. (C) Image of a 1% agarose gel following electrophoresis, which represent the various sizes of DNA following EcoRI digestion.

NUDC-FANCD2 Interaction in Situ

Subsequent to cloning NUDC, we aimed to purify the 1.2 kb band of NUDC (Figure 2.7A), which contained the NUDC gene based upon our construct map (Figure 2.6B). The RPF-vector was also cut, and both the RPF-vector and NUDC plasmids were purified and detected via agarose gel electrophoresis (Figure 2.7B). Once this was achieved, we ligated our RFP-vector with NUDC and successfully cloned RFP-NUDC. Following this, we picked up eight colonies and performed miniprep to extract the RFP-NUDC plasmid DNA. EcoRI was used to cut our samples, whereby, samples 2-8 expressed our band of interest (Figure 2.7C). Subsequently, one of the correctly identified miniprep samples was then cultured and midiprep was performed.

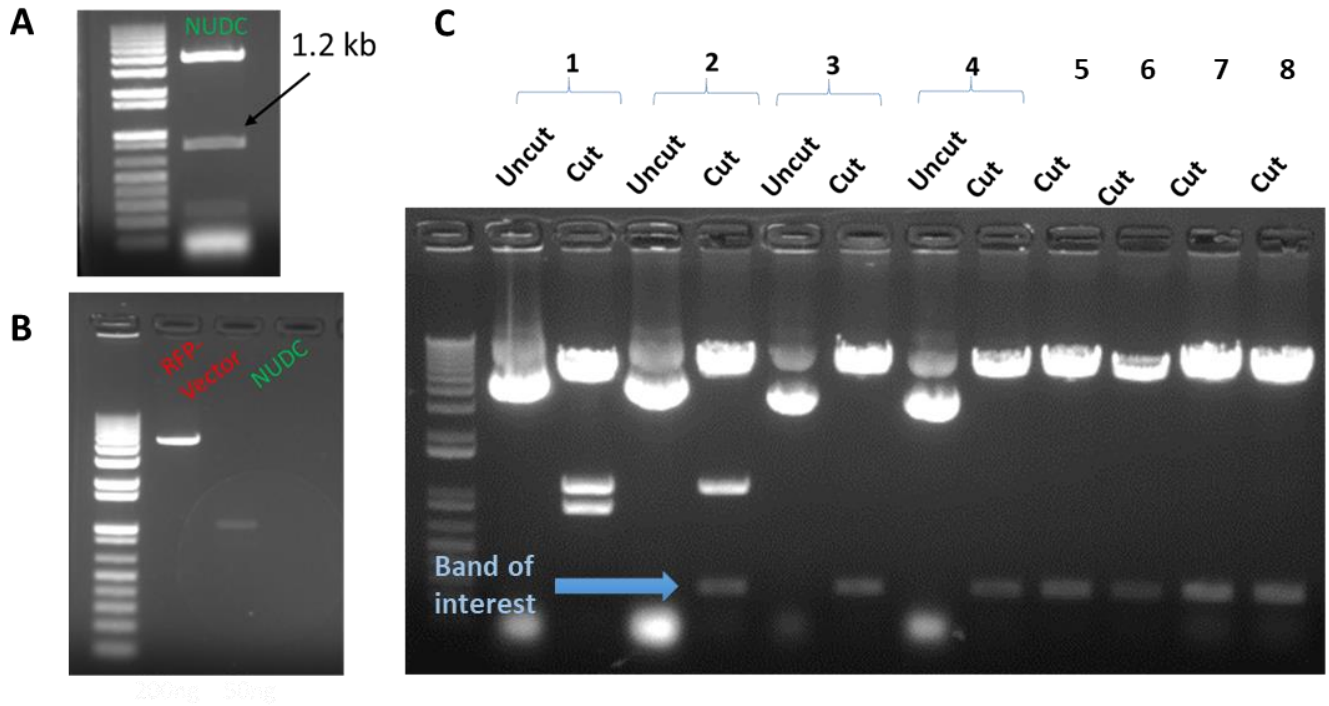


Figure 2.7 RPF-NUDC purification and ligation detected via agarose gel electrophoresis. (A) Identification of the 1.2 kb band that contained the NUDC gene following EcoRI digestion. (B) RFP and NUDC purified and confirmed by their size (C) Miniprep samples following RFP-NUDC ligation and digested by the restriction enzyme HinIII. Blue arrow represent band of interest.

Following midiprep, the RFP-NUDC plasmid was transfected into 293T cells and imaged via a fluorescent microscope to detect the transfection efficiency (Figure 2.8A). In addition, the transfection efficiency was confirmed via WB (Figure 2.8B).

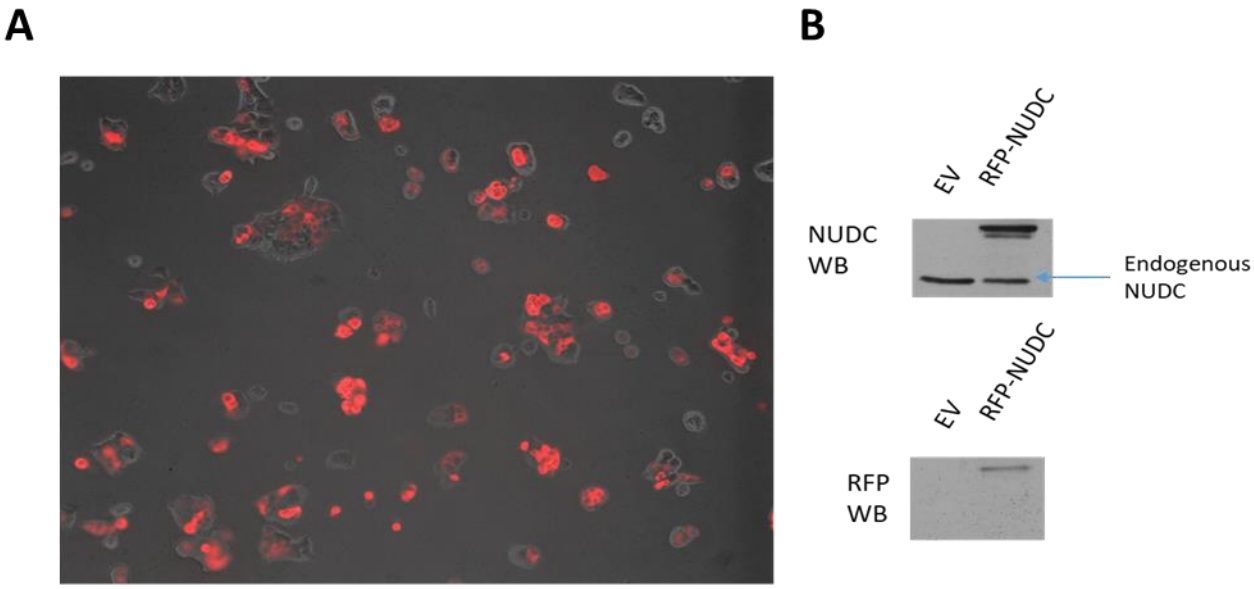


Figure 2.8 RFP-NUDC transfection into 293T cells. (A) Transfection efficiency observed under a fluorescent microscope, RFP fluoresces red. (B) WB confirmation of transfection efficiency. NUDC is detected at ~43 kDa in both EV and RFP-NUDC transfection samples, however, in the RFP-NUDC sample a second band is also detected, which represents the NUDC that was transfected into the cell. RFP is only detected in the RFP-NUDC transfected sample.

Concurrently, we obtained GFP-EV and GFP-FANCD2 plasmid samples from our lab and transfected them into 293T cells. Following this, we observed our cells under a fluorescent microscope to ensure an appropriate transfection efficiency (Figure 2.9). Initially, transfecting the GFP-wtFANCD2 plasmid into the 293T cells was not effective. To troubleshoot this, we cut GFP-wtFANCD2 with Cla1 and purified the plasmid prior to the transfection. Despite this, the transfection efficiency was still higher in GFP-EV cells compared to GFP-WtFANCD2.

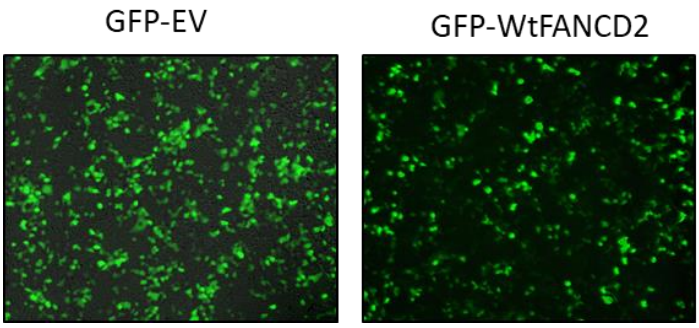


Figure 2.9 GFP-EV and GFP-wtFANCD2 transfection efficiency in 293T cells. The GFP tag in both EV and wtFANCD2 transfected cells exhibit a green fluorescence under the fluorescent microscope.

Finally, to determine *in situ* interaction between FANCD2 and NUDC, we performed a co-transfection and transfected both our GFP-wtFANCD2 plasmid and RFP-NUDC plasmid into 293T cells. This experiment successfully demonstrated that FANCD2 and NUDC interact using live fluorescent imaging, which determined an overlap in the cells expression of green and red to exhibit orange fluorescent cells (Figure 2.10).

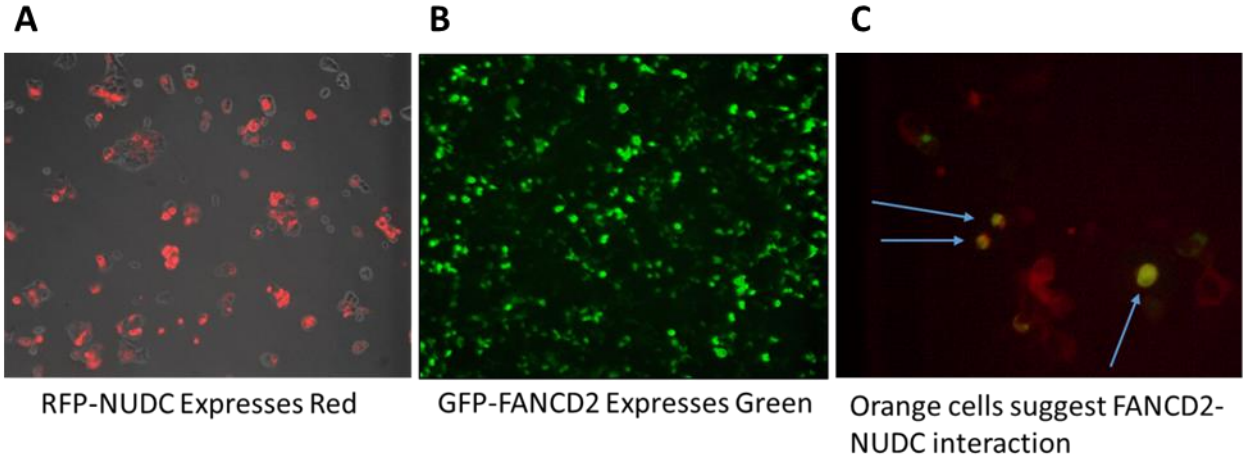


Figure 2.10 Fluorescent imaging suggests interaction between FANCD2 and NUDC. (A) RFP tag protein expressed in the cells exhibit red fluorescence. (B) GFP tag protein expressed in the cells exhibit green fluorescence. (C) Cells co-transfected with GFP-wtFANCD2 and RFP-NUDC observed

under live imaging. Blue arrows represent cells exhibiting a combination of a green and red (orange) fluorescence.

Exogenous FANCD2-NUDC IP

To further validate the previous observations between FANCD2 and NUDC interaction, we co-transfected wtFANCD2, which contains a flag-tag protein, and RFP-NUDC into 293T cells. From this experiment, we detected Flag-FANCD2 pulldown in NUDC IP and Flag IP samples, as well as NUDC pull-down in Flag IP and NUDC IP samples (Figure 2.11). Together, this continues to support interaction between the FANCD2 and NUDC.

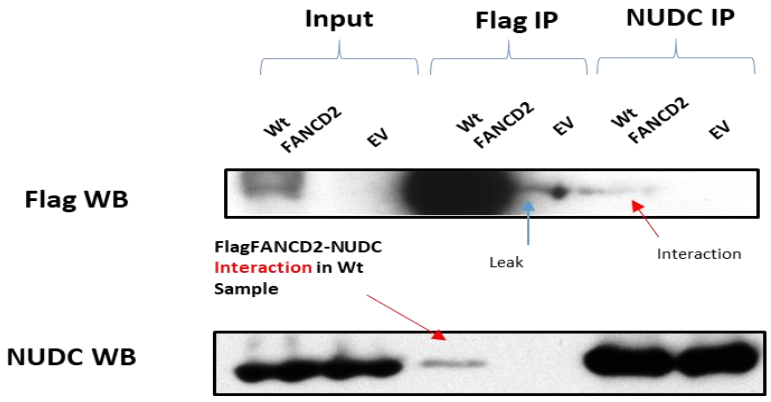


Figure 2.11 Exogenous FANCD2-NUDC IP. Confirmation of exogenous protein interaction between transfected NUDC and wtFANCD2 containing a Flag tag protein.

The biological effect of FANCD2 and NUDC in chromosomal segregation

Previously, we identified that FANCD2 knockdown promoted abnormal chromosomal segregation in cells following IR via FACS analysis. Following IR, control cells were not observed to contain cells with abnormal octaploids (8N) until 24 h post IR, which decreased at 36 h post IR. However, in FANCD2 knockdown cells, immediately after IR, cells exhibiting 8N were observed as comparable to control cells 24 h post IR. In FANCD2 knockdown cells, a similar trend was observed, whereby, cells containing 8N peaked at 24 h post IR and this was reduced at 36 h post

IR. However, compared to their control counterparts, FANCD2 knockdown cells exhibited a much higher number of cells, which contained 8N (Figure 2.12).

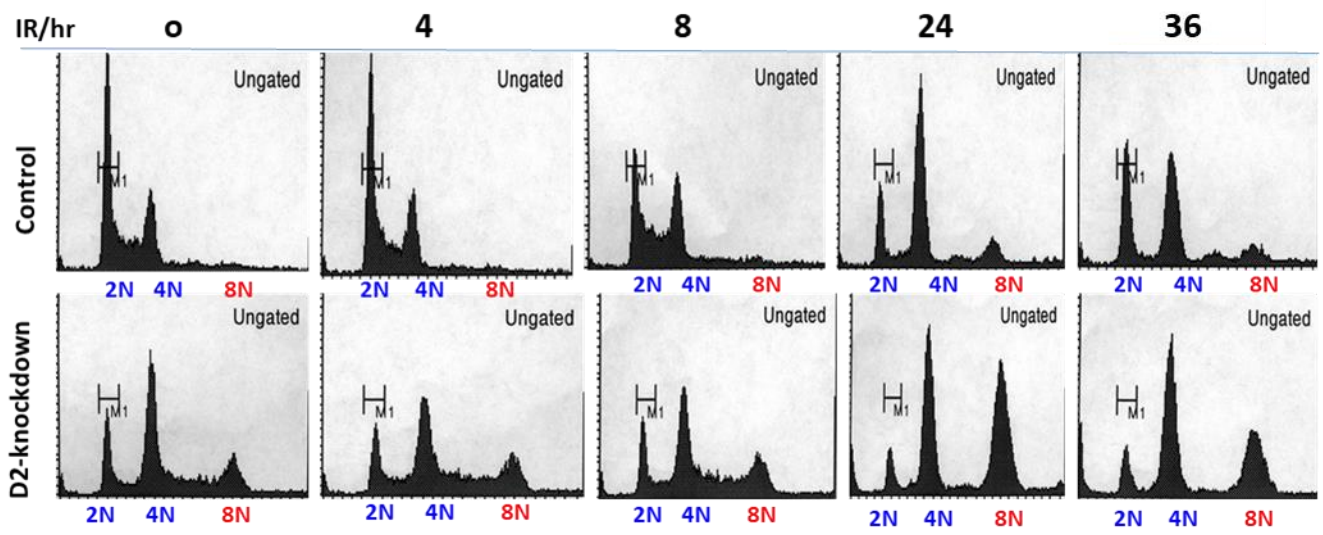


Figure 2.12 FACS analysis of control and FANCD2 knockdown cells post IR. 2N represents a diploid cell, which contains a single set of chromosomes. 4N represents a haploid cell containing 2 sets of chromosomes. 8N represents an octoploid cell, which is abnormal and indicates dysfunction in M phase.

Similarly, as NUDC is involved in M-phase processes [127, 137] and is a binding partner of FANCD2 we wanted to determine whether they performed similar biological functions with respect to chromosomal segregation. Therefore, via a lentivirus delivery system we aimed to silence NUDC. To achieve this, we first ligated our NUDC oligo with the pLKO.1 TRC-cloning vector and transformed the ligation mixture into competent bacteria. Five colonies were picked up and cultured, before performing miniprep. Following this, EcoRI and NBEI were used to digest our samples, through which samples 1, 4 and 5 expressed our band of interest (Figure 2.13A). Similarly, sample 1 was sent off for sequencing, which identified a 100% match to our oligo template (sequence 1) (Figure 2.13B). Thus, sample 1 was further cultured and midiprep was

performed. Likewise, EcoR1 and NBE1 was used to cut the sample and agarose gel electrophoresis identified the same band of interest (Figure 2.13C).

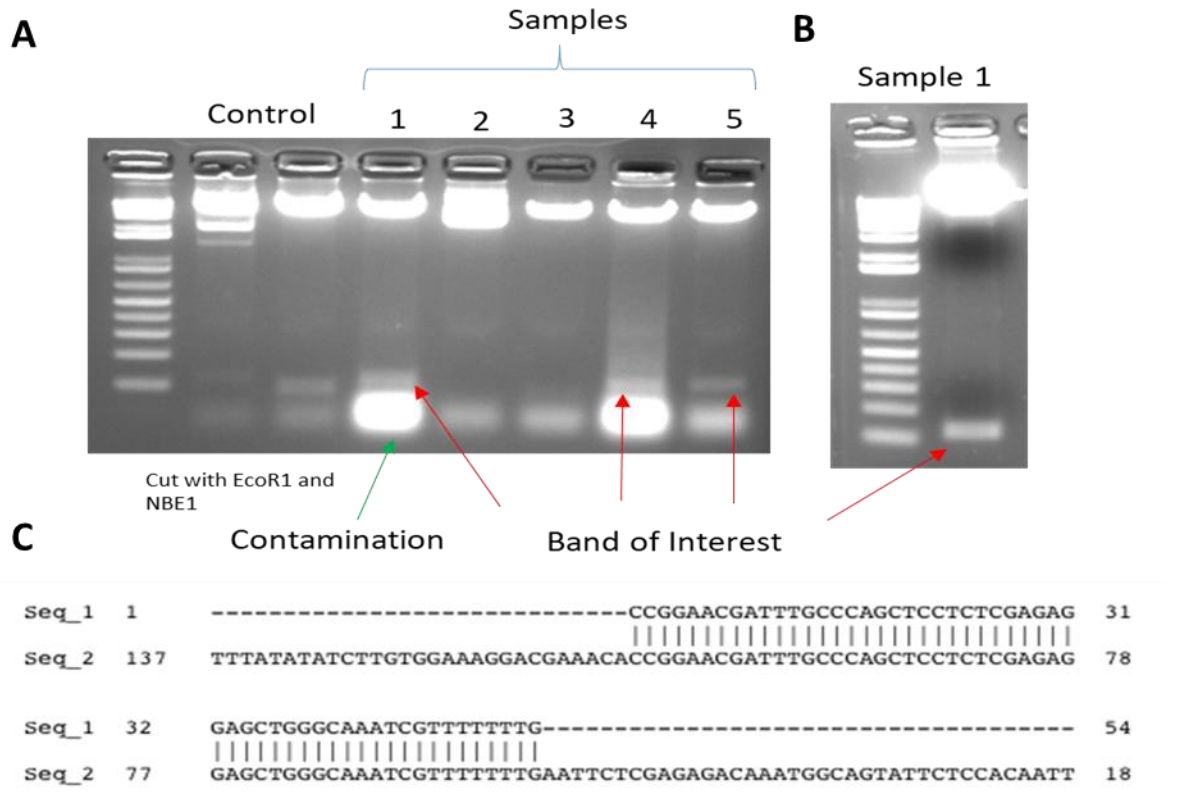


Figure 2.13 pLKO.1-NUDC ligation. (A) Miniprep samples of potential pLKO.1-NUDC colonies cut with EcoR1 and NBE1. (B) Midiprep sample of Sample 1 cut with EcoR1 and NBE1. (C) Sample 1 set for sequencing, to confirm correct pLKO.1-NUDC ligation and plasmid.

As described above, we infected U2OS cells with a lentivirus, designed to silence NUDC. Cells infected with a 1/1 titer of the lentivirus had very high levels of cell death and did not continue to proliferate. Therefore, we diluted our infection medium to 1/2, 1/5, and 1/10 titer. Puromycin treatment at 1 µg/µl for 1-2 w confirmed U2OS cells were infected using the puromycin selection marker, according to the pLKO.1 puro with shRNA construct map (Figure 2.14A). Following our transfection of the 293T cells, these cells were immediately collected. WB analysis demonstrated

that NUDC expression was lower in these cells compared to the EV control infected cells. However, stable U2OS cells infected with the lentivirus targeting NUDC expressed lower levels of NUDC via WB compared to EV control and transiently transfected 293T cells (Figure 2.14B).

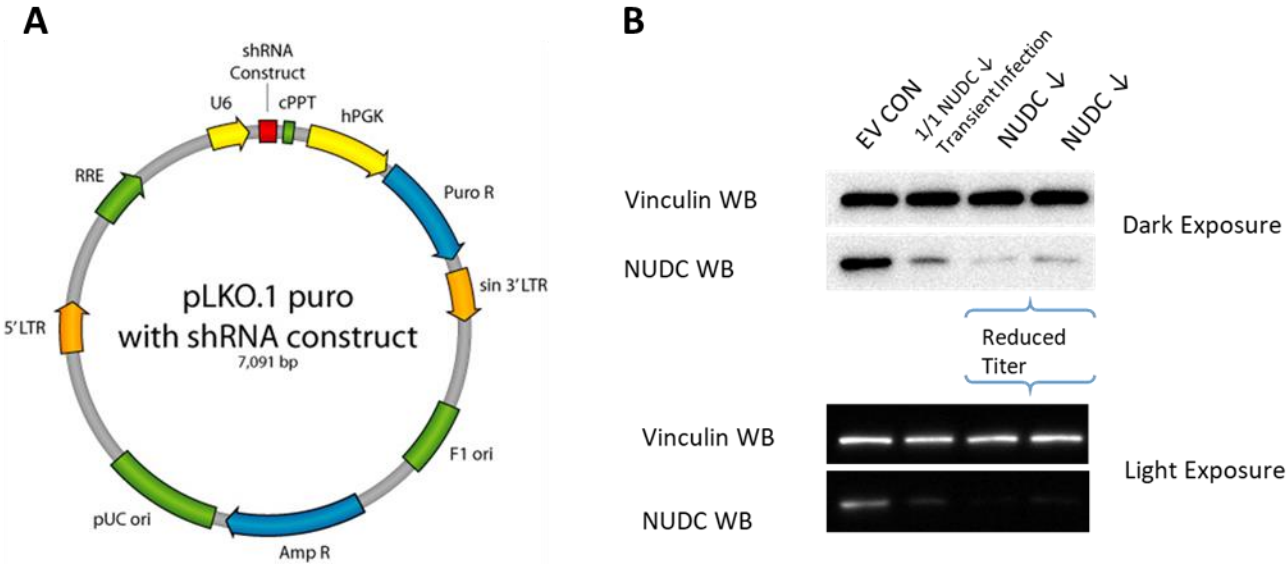


Figure 2.14 Lentiviral transduction of pLKO.1 plasmids containing shRNA targeting NUDC. (A) Construct maps of pLKO.1 including puromycin resistance (B) WB detecting NUDC confirmed the success of NUDC knockdown via lentiviral transduction.

Next, using the stable EV control and NUDC↓ cells we performed FACS analysis on cells treated with cisplatin compared to control. Although we did not observe significant differences between our conditions, a minor peak was observed in our sample 1/10 NUDC 36 h post cisplatin treatment (Figure 2.15A). However, further analysis of our results using a different channel we observed similarities in the 1/10 NUDC↓ cells similar to that of FANCD2↓ cells, following DNA damage (Figure 2.15B).

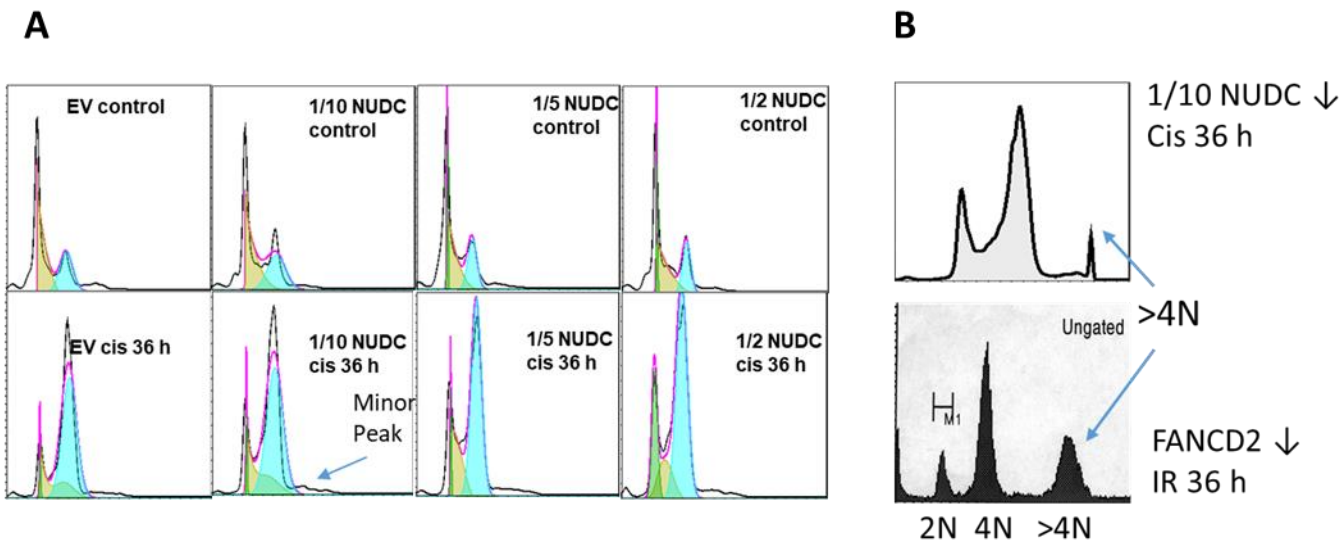


Figure 2.15 FACS analysis of U20S cells following DNA damage. (A) EV and NUDC knockdown cells were treated with cisplatin for 36 h and compared to untreated control cells via FACS analysis. (B) NUDC knockdown cells treated with cisplatin are compared to FANCD2 knockdown cells 36 h post IR via FACS analysis.

In addition to investigating the role of NUDC on chromosomal segregation, we observed that cells infected with the lentivirus targeting NUDC proliferated at a slower rate initially, compared to the EV control cells. Therefore, we investigated the effects of growth via a proliferation assay, however, no significant differences were observed.

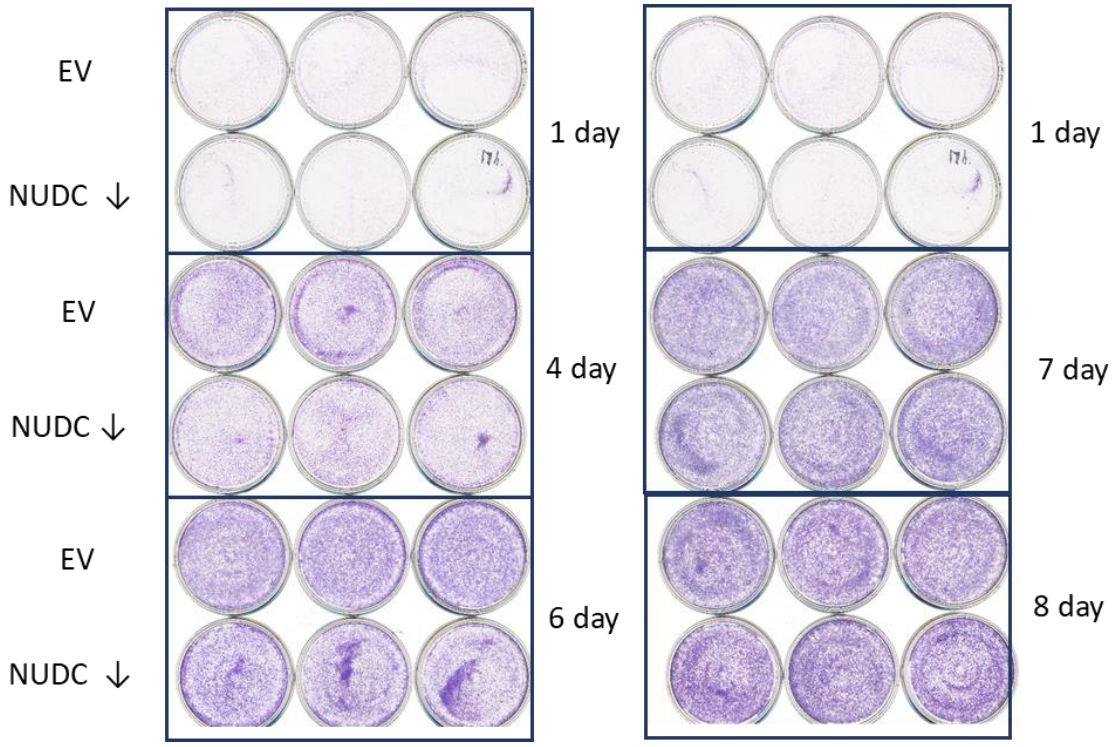


Figure 2.16 Proliferation assay in EV control cells compared to NUDC↓ cells.

2.5 Discussion

To better understand the role of FA signaling in tumorigenesis, we investigated the role of FA signaling in the regulation of M-phase. With the emergence of many new functions, it is now clear that FA signaling is far more complex than previously thought [144]. This confirms previous hypotheses made that an impaired response to DNA damage does not fully account for the FA phenotype, or its associated symptoms [144]. Therefore, further investigation is required in order to understand how FA signaling promotes genome stability and govern numerous biological processes throughout the lifespan. FA signaling largely acts to prevent the accumulation of genetic mutations during interphase, which includes G_1, S and G_2 [145]. However, our findings support the role of FA signaling, as a regulator of cell division. In addition, previous studies have observed that numerous FA proteins localize to important sites during cell division such as the centrosome and mitotic spindles [101].

Here, we report that FANCD2 interacts with the M-phase protein NUDC. Using MS/MS mass spectroscopy, we conducted an unbiased screening of FANCD2 and its functional units. Following this, a number of proteins were identified to associate with FANCD2, which included NUDC (Figure 2.3). To validate this interaction we first performed gel filtration analysis and found that in vector and slow cells, FANCD2 and NUDC shared similar peak expressions (Figure 2.4). This suggested that both proteins, along with their binding partners share a similar complex size, which would support interaction. Next, we performed numerous IP experiments and confirmed both endogenous (Figure 2.5) and exogenous interaction (Figure 2.11). We found that FANCD2 antibodies pulled-down NUDC, and correspondingly NUDC antibodies were capable of pulling down FANCD2. Following this, we cloned NUDC based upon sequences provided by NCBI, and ligated NUDC with RPF. Next, using the GFP-NUDC plasmid, which was previously created in our lab, we performed a co-transfection and identified FANCD2-NUDC interaction *in situ* (Figure 2.10).

Once we validated the interaction between FANCD2 and NUDC, we next sought to investigate the functional significance. The cell system we previously designed involved cells with reduced FANCL activity. FANCL acts as the catalytic subunit to monoubiquitinate the FA ID complex [6]. Therefore, this unique system represents the inactivation of FANCD2 *in vitro* without using knockdown/knockout models. Initially, these FANCL↓ cells were labelled slow cells. As their name suggests, the slow cells proliferated at a slower rate compared to the CRL vector cells. Upon two and three years of continual passaging, we created medium and fast cells, respectively. This discovery alone provides insight in a potential role of FA signaling in proliferation, however, the rapid growth may also represent the progression towards a cancer phenotype. Interestingly, FANCD2 was observed to interact the most with NUDC in slow cells, where this interaction was progressively weakened in medium, fast, and vector cells (Figure 2.5). Further investigation will be required to understand this phenomenon, as our current data is somewhat unclear.

As well as abnormal proliferation rates, we also identified abnormalities in FANCD2 knockdown cells following IR (Figure 2.12). The detection of a high number of octoploid cells containing 8N eludes to a number of roles for FANCD2 in M-phase. FANCD2 has been previously reported to act

as a spindle assembly checkpoint protein to arrest mitosis [145]. Alternatively, FANCD2 may also promote chromosome segregation following IR in order to replace damaged or apoptotic cells. Failure to successfully segregate duplicated chromosomes into separate cells represents a defect in either mitosis and/or cytokinesis, which leads to aneuploidy, a characteristic often observed in cancerous cells [151].

With the newfound discovery that NUDC interacts with FANCD2, we sought to establish whether the same consequences could be observed in a NUDC knockout/knockdown model. To achieve this, we infected U2OS cells *in vitro*, using a lentivirus delivery system to silence NUDC. Although, it was unclear whether these cells expressed NUDC at the protein level, the full concentration of the lentivirus infection lead to cell lethality, which was not observed in our EV control cells. This observation supports previous findings that suggest NUDC is essential for cell viability [119]. Simultaneously, we also infected cells with the same virus at a reduced viral titer. Following this, we observed that NUDC knockdown cells appeared to proliferate at a slower rate compared the EV control cells. However, when we completed our proliferation assay we were unable to observe any differences between our EV and NUDC knockdown cells (Figure 2.16). Finally, we did not observed the same effects in NUDC knockdown cells treated with cisplatin, a DNA crosslinking agent, compared to the irradiated FANCD2 knockdown cells (Figure 2.15A). Although, upon further analysis of our data we identified a similar trend amongst these conditions (Figure 2.15B). Therefore, we believe that FANCD2 may partly regulate error-free chromosomal segregation via its interaction with NUDC, however, further research is required to determine the functional significance of their interaction.

In summary, this study provided evidence to add to the body knowledge, which suggests that FA signaling promotes the regulation of M phase. Here, we proposed that NUDC is a target of FA signaling to regulate the cell cycle. Although, we were unable to demonstrate the biological effects responsible by the interaction between FANCD2 and NUDC, we have provided sufficient data to clearly demonstrate their interaction. Furthermore, this study lays out a strong foundation to elucidate a novel mechanism(s) by which FANCD2 regulates cell division.

Chapter Three: Future Direction

3.1 Future and Ongoing Work

In this project we successfully cloned two novel isoforms of NUDC, which has not been previously performed (Figure 2.6). However, NCBI has identified an additional three isoform of the NUDC gene present in humans, which have yet to be clone. This research could lead to novel discoveries in the role of NUDC, as well as expand our findings by which FANCD2 interacts with NUDC. Previously, we have reported that FANCD2 exists in two variants, FANCD2-V1 and FANCD2-V2 [152]. These two variants indicate distinct functions with respect to tumorigenesis [152], therefore, we have begun investigating whether or not NUDC preferentially binds to either FANCD2 isoform (Figure 3.1).

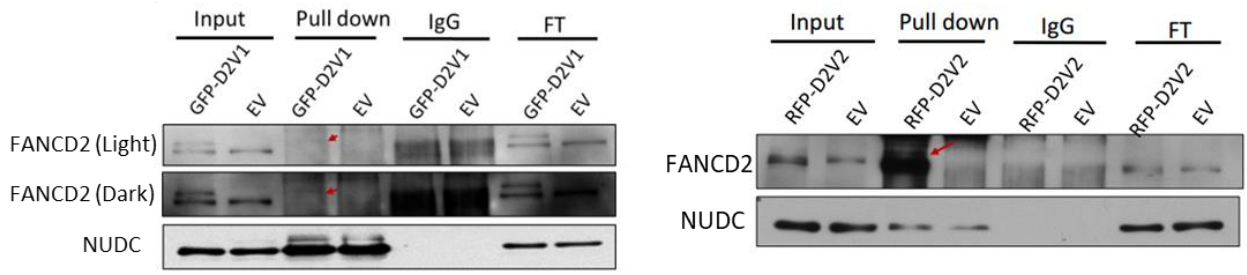


Figure 3.1 FANCD2-V1-NUDC and FANCD2-V2-NUDC IP in Transfected 293T cells. GFP-D2V1 represents cells transfected with GFP-FANCD2-V1 and RFP-D2V2 represents cells transfected with RFP-FANCD2-V2. Red arrows highlight pulldown (interaction).

Furthermore, through bioinformatics and protein docking analysis we have determined five potential sites of FANCD2 and NUDC interaction (Figure 3.2).

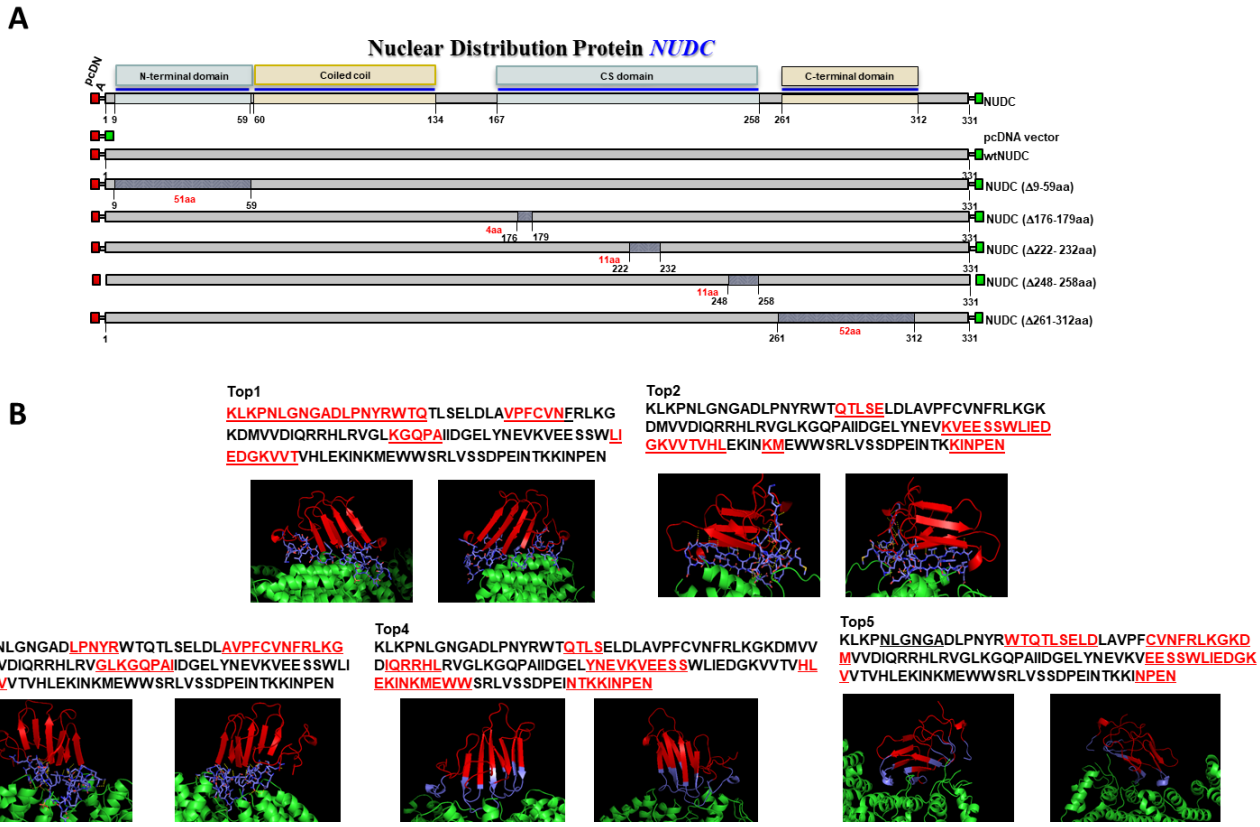


Figure 3.2. FANCD2 is predicted to interact with NUDC amongst five possible regions. (A) Schematic representation of a series of deletions in NUDC, where FANCD2 may bind. (B) The molecular docking of FANCD2 and NUDC at the potential sites of interaction.

Based upon these predictions, we designed five NUDC cDNA plasmids, which encode for wtNUDC and five mtNUDC proteins containing the deleted motifs 1-5, respectively. Using mutagenesis PCR to delete various AA regions in NUDC, we performed similar experiments outlined above in our study. To analyze the location of FANCD2-NUDC interaction we performed IF imaging of 293T cells co-transfected with GFP-FANCD2 and RFP-NUDC, and RFP-NUDC mutants 1-5 (Figure 3.3).

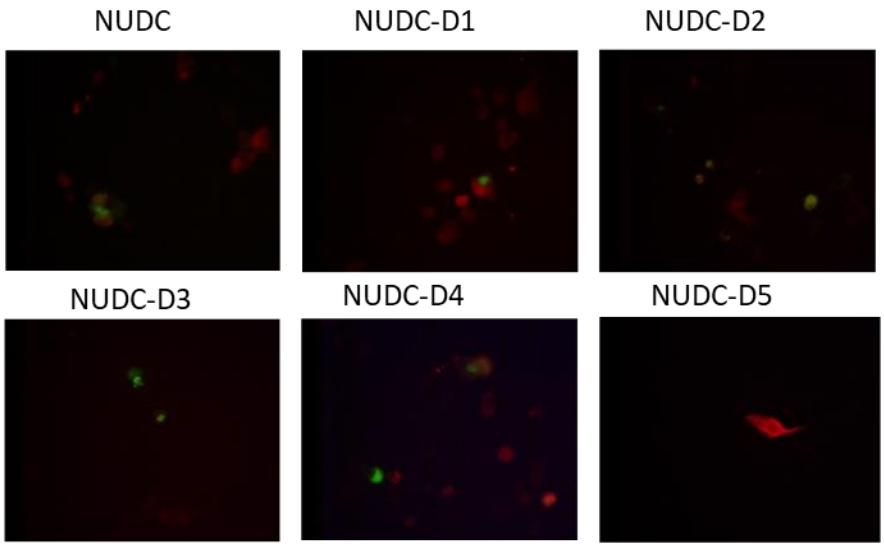


Figure 3.3. Immunofluorescence to highlight FANCD2-NUDC interaction. D1-D5 represents deletion 1-5 respectively. Green fluorescence represents GFP-FANCD2. Red fluorescence represents RFP-NUDC.

Here, we have summarized the ongoing work described in our study to complete this project. In addition, to the characterization of the region by which NUDC interacts with FANCD2, we intend to demonstrate the biological effects of this interaction. This will be achieved by developing cell systems based upon the mtNUDC deletion plasmids. Once stable cells are developed, we will utilize xenograft models to investigate the role of FANCD2-NUDC interaction in tumorigenesis.

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